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The Journal of
The American Society of
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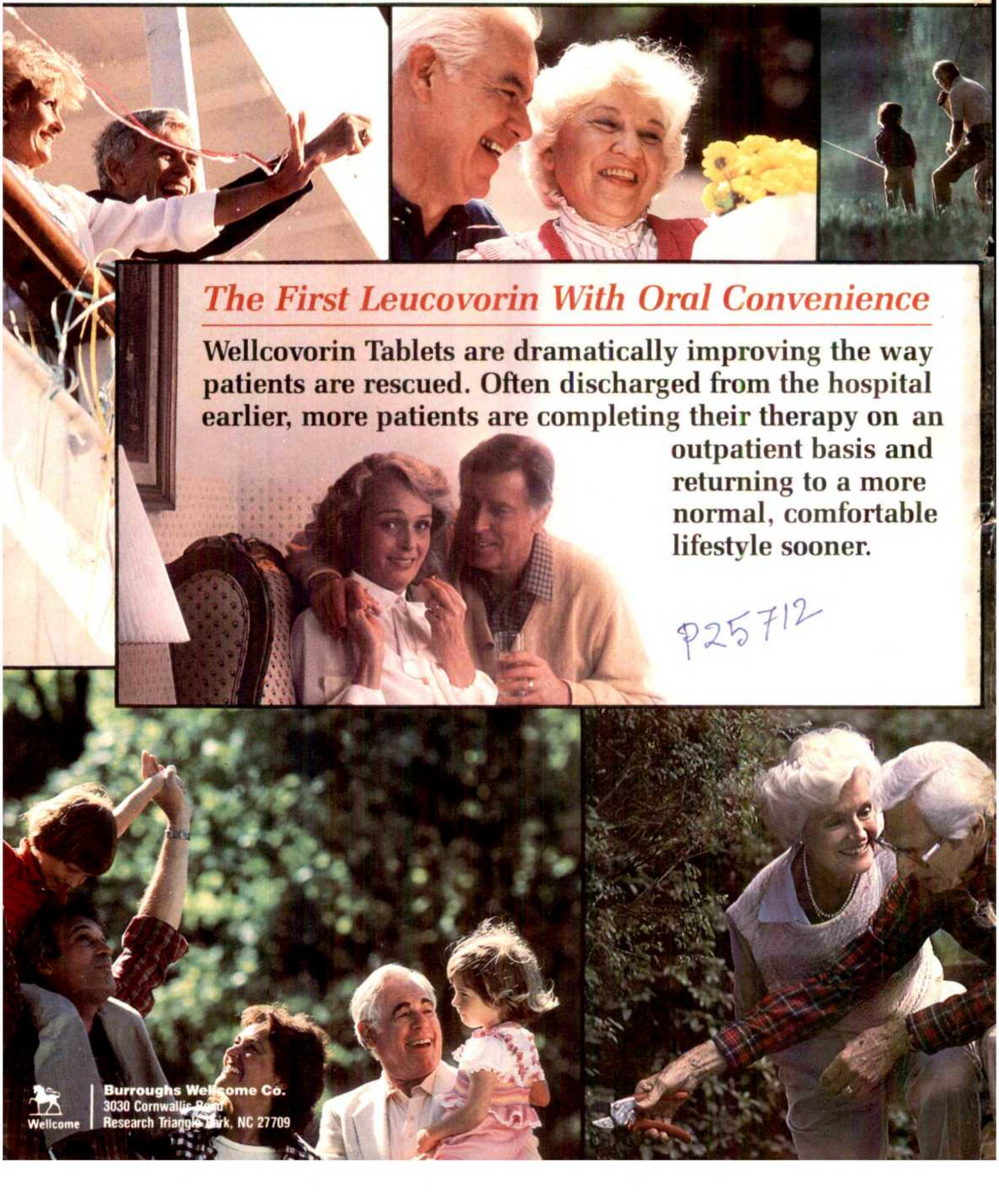

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[1] Blum, Guaspari, et al: NCI Folate Symposium, Jan. 1986.

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Wellcovorin Tablets have reduced the cost of leucovorin rescue significantly. Before Wellcovorin Tablets, the cost of leucovorin increased rapidly and consistently every year. Now, patients pay less for Wellcovorin Tablets, and often realize considerable savings from a reduction in hospital stays and clinic visits.

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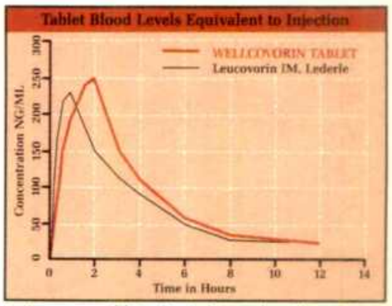
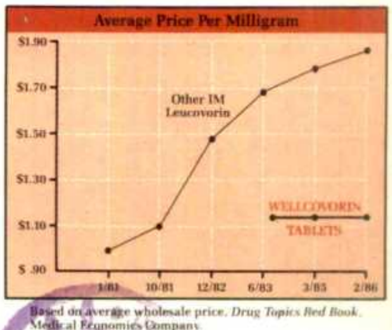


Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al.

Blum MR, Guaspari A, Liao SHT, et al: Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm. Sci. San Diego, California. 1982. 12(2):175.



Based on average wholesale price. *Drug Topics Red Book*, Medical Economics Company.

WELLCOVORIN® TABLETS

(leucovorin calcium)

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Please see brief summary of prescribing information on next page.

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WELLCOVORIN® TABLETS
(leucovorin calcium)
Leucovorin in convenient
5 mg and 25 mg tablets

Before prescribing WELLCOVORIN® Tablets, please consult complete prescribing information. The following is a brief summary.

INDICATIONS AND USAGE: Wellcovorin (leucovorin calcium) is indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: ***Teratogenic Effects:*** Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below $5 \times 10^{-8}M$.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer*, *41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al*: High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med*, 68:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro*. *Br J Haematol*, *40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al*: The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood*, 55(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis*, *135*(3): 493-496, 1977.

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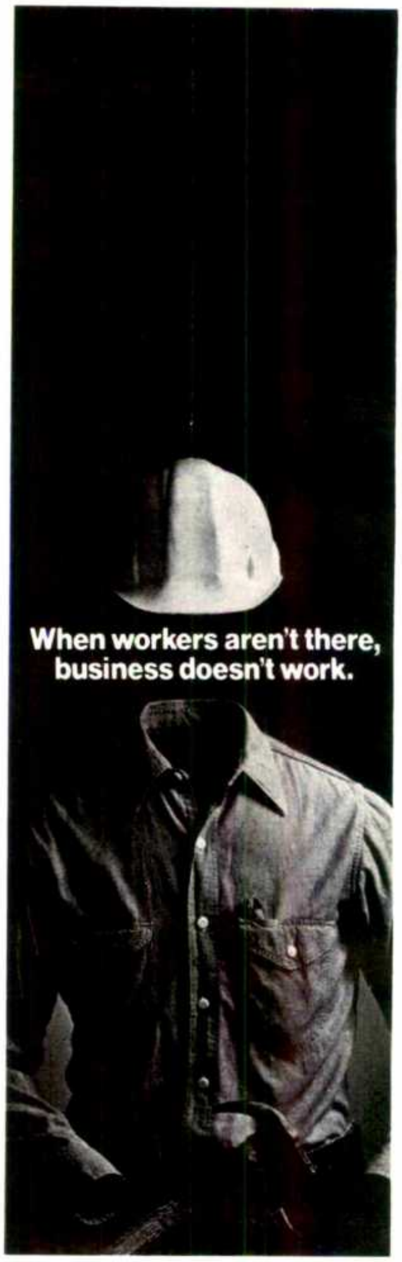
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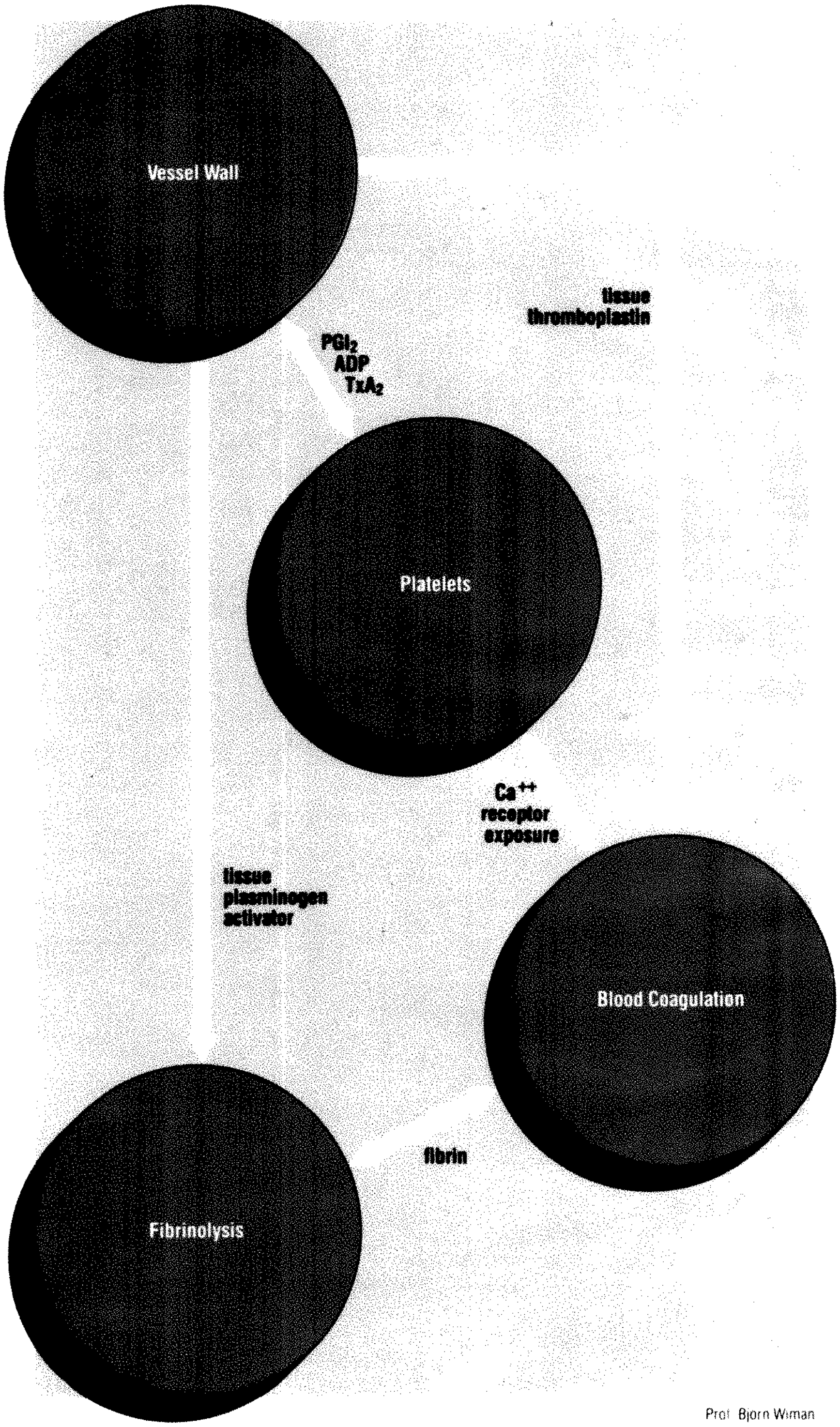

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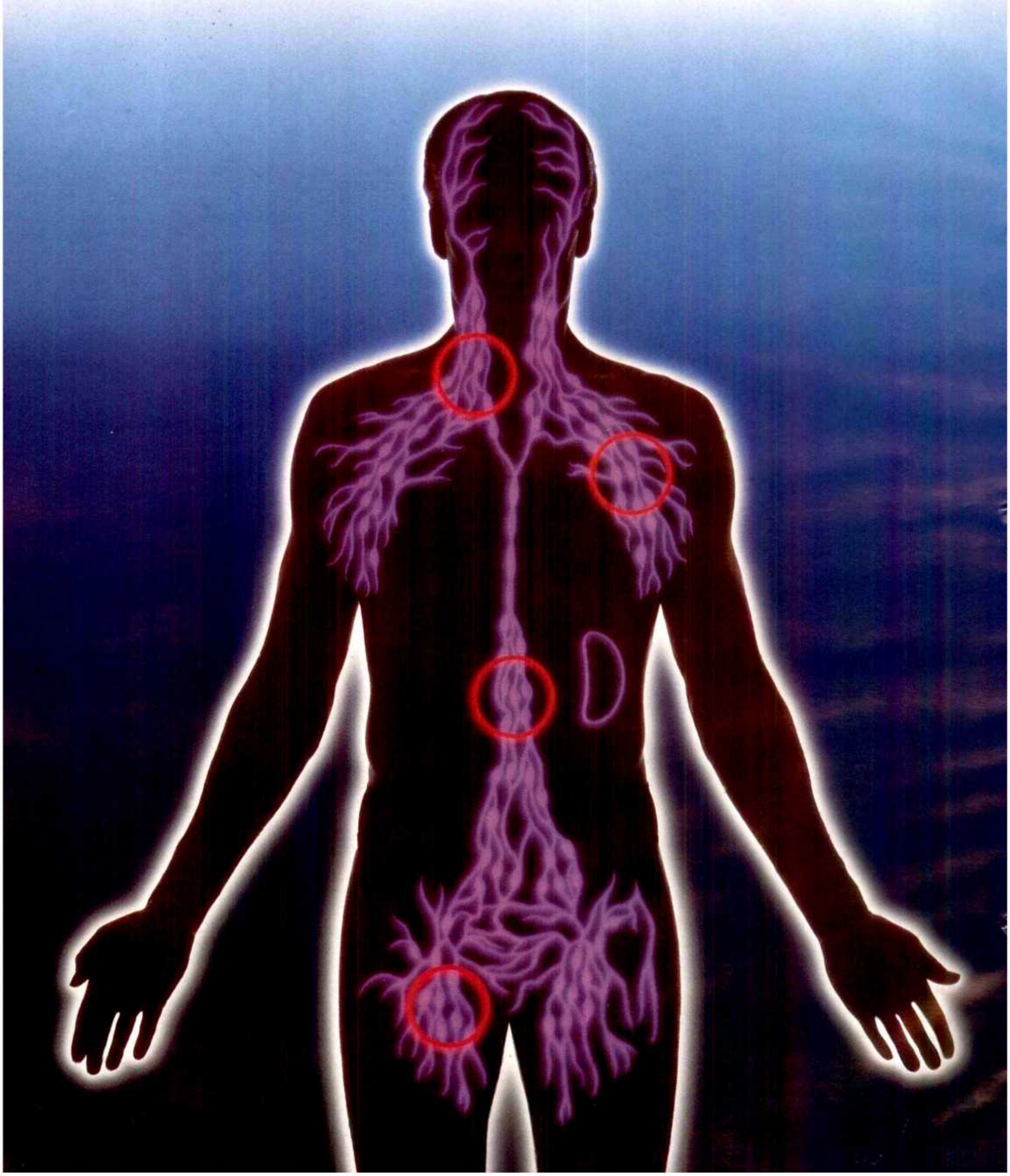
Aggressive Therapy for



Aggressive Lymphomas

Approaching a cure for non-Hodgkin's lymphomas (NHL)

Lymphomas were considered incurable a scant two decades ago; now, cure is a reality for as many as half of all patients with NHL.[1] The frequency of complete remissions—the most critical factor influencing prognosis—has increased, and long-term disease-free survival is an attainable goal for many patients.

With innovative therapeutic programs

Combination chemotherapy now serves as a primary therapy for the vast majority of patients with intermediate-to-poor prognosis NHL. Treatment strategies depend on many factors, including age, histology, and stage of the primary disease, and presence of comorbid disease. Consequently, no one therapeutic program can be cited as best. Fortunately, the availability of multiple non-cross-resistant regimens has permitted individualization of therapy.

Methotrexate...a component of aggressive regimens improving the outcome in NHL

Lederle introduced Methotrexate in the 1950s. Since then, Methotrexate has been used in many different ways, reflecting the changing approaches to clinical oncology. One important application is in the treatment of NHL.

1. DeVita VT Jr, Hellman S: Hodgkin's disease and the non-Hodgkin's lymphomas, in DeVita VT Jr, Hellman S, and Rosenberg SA (eds): *Cancer, Principles and Practice of Oncology*. Philadelphia, J. B. Lippincott Company, 1982, p. 1392.

Please review the brief summary of prescribing information on the next page

LEDERLE

METHOTREXATE

METHOTREXATE
Brief Summary

WARNINGS:

WARNING
METHOTREXATE MUST BE USED ONLY BY PHYSICIANS EXPERIENCED IN ANTIMETABOLITE CHEMOTHERAPY. BECAUSE OF THE POSSIBILITY OF FATAL OR SEVERE TOXIC REACTIONS, THE PATIENT SHOULD BE FULLY INFORMED BY THE PHYSICIAN OF THE RISKS INVOLVED AND SHOULD BE UNDER HIS CONSTANT SUPERVISION. DEATHS HAVE BEEN REPORTED WITH THE USE OF METHOTREXATE IN THE TREATMENT OF PSORIASIS.

IN THE TREATMENT OF PSORIASIS, METHOTREXATE SHOULD BE RESTRICTED TO SEVERE RECALCITRANT, DISABLING, PSORIASIS WHICH IS NOT ADEQUATELY RESPONSIVE TO OTHER FORMS OF THERAPY, BUT ONLY WHEN THE DIAGNOSIS HAS BEEN ESTABLISHED, AS BY BIOPSY AND/OR AFTER DERMATOLOGIC CONSULTATION.

1. Methotrexate may produce marked depression of bone marrow, anemia, leukopenia, thrombocytopenia, and bleeding.
2. Methotrexate may be hepatotoxic, particularly at high dosage or with prolonged therapy. Liver atrophy, necrosis, cirrhosis, fatty changes, and periportal fibrosis have been reported. Since changes may occur without previous signs of gastrointestinal or hematologic toxicity, it is imperative that hepatic function be determined prior to initiation of treatment and monitored regularly throughout therapy. Special caution is indicated in the presence of preexisting liver damage or impaired hepatic function. Concomitant use of other drugs with hepatotoxic potential (including alcohol) should be avoided.
3. Methotrexate has caused fetal death and/or congenital anomalies; therefore, it is not recommended in women of childbearing potential unless there is appropriate medical evidence that the benefits can be expected to outweigh the considered risks. Pregnant psoriatic patients should not receive Methotrexate.
4. Impaired renal function is usually a contraindication.
5. Diarrhea and ulcerative stomatitis are frequent toxic effects and require interruption of therapy; otherwise hemorrhagic enteritis and death from intestinal perforation may occur.

METHOTREXATE HAS BEEN ADMINISTERED IN VERY HIGH DOSAGE FOLLOWED BY LEUCOVORIN RESCUE IN EXPERIMENTAL TREATMENT OF CERTAIN NEOPLASTIC DISEASES. THIS PROCEDURE IS INVESTIGATIONAL AND HAZARDOUS.

INDICATIONS: Antineoplastic Chemotherapy: Methotrexate is indicated for treatment of gestational choriocarcinoma, in patients with chorioadenoma destruens and hydatidiform mole; palliation of acute lymphocytic leukemia, treatment and prophylaxis of meningeal leukemia, and palliation of acute lymphoblastic (stem-cell) leukemias in children. May be used for induction of remission in combination with other anticancer drugs or suitable agents, but is most commonly used in maintenance of induced remissions. May be used alone or in combination with other anticancer agents in management of breast cancer, epidermoid cancers of the head and neck, and lung cancer, particularly squamous cell and small cell types. Also effective in treatment of advanced stages of lymphosarcoma, particularly in children, and in advanced cases of mycosis fungoides. See box warnings re *Psoriasis Chemotherapy;* because of high risk Methotrexate is indicated only in symptomatic control of severe, recalcitrant, disabling psoriasis which is not adequately responsive to other forms of therapy, *only when diagnosis has been established, as by biopsy and/or after dermatologic consultation.*

CONTRAINDICATIONS: Psoriatic patients who are pregnant, or have severe renal or hepatic disorders; or have preexisting blood dyscrasias such as bone marrow hypoplasia, leukopenia, thrombocytopenia, or anemia, should not receive Methotrexate.

PRECAUTIONS: Methotrexate has high potential toxicity, usually dose-related; physicians should be familiar with various characteristics and its established clinical usage. Subject patients to appropriate supervision to detect and evaluate signs or symptoms of possible toxic effects or adverse reactions with minimal delay. Hematopoietic suppression is a common effect of Methotrexate; pretreatment and periodic hematologic studies are essential to its use in chemotherapy. This effect may occur abruptly and on apparently safe dosage; any profound drop in blood-cell count indicates immediate stopping of drug and appropriate therapy. Use drug with caution, if at all, in patients with malignant disease who have preexisting bone marrow aplasia, leukopenia, thrombocytopenia, or anemia.

Methotrexate is excreted principally by the kidneys; use in the presence of impaired renal function may result in accumulation of toxic amounts or additional renal damage. Renal status should be determined prior to and during therapy; exercise proper caution should significant renal impairment be disclosed. Reduce or discontinue drug dosage until renal function is improved or restored.

The following laboratory tests are recommended as part of essential clinical evaluation and appropriate monitoring of patients to determine any existing organ dysfunction or system impairment: complete hemogram, hematocrit, urinalysis, renal function and liver function tests, and chest x-ray. Perform tests prior to, at appropriate periods during, and after termination of therapy. Where high dose or long-term therapy is followed, performance of liver biopsy or bone marrow aspiration studies may be useful or important. Methotrexate is bound in part to serum albumin after absorption; toxicity may be increased because of displacement by drugs such as salicylates, sulfonamides, diphenylhydantoin, phenylbutazone; by antibacterials such as tetracycline, chloramphenicol and para-amino-benzoic acid. Do not give these drugs concurrently, especially salicylates, phenylbutazone, and sulfonamides, whether antibacterial, hypoglycemic or diuretic, until significance of these findings is established. Vitamin preparations containing folic acid or its derivatives may alter responses to Methotrexate. Use with extreme caution in presence of infection, peptic ulcer, ulcerative colitis, debility, and extreme youth or old age. Bacterial infection may occur or become a threat if profound leukopenia occurs during therapy; cessation of drug and appropriate antibiotic therapy is usually indicated. Blood or platelet transfusions may be necessary in severe bone marrow depression. Methotrexate may have an immunosuppressive action; take into consideration when immune responses in a patient may be important or essential. In all instances, physician must evaluate need and usefulness of drug against risk of toxic effects or adverse reaction; most adverse reactions are reversible if detected early. Reduce dosage or discontinue drug when such effects or reactions do occur; take appropriate corrective measures according to clinical judgement of physician. Reinstitute therapy with caution, consideration of further need for drug, and alertness to possible toxicity recurrence.

ADVERSE REACTIONS: Most common include ulcerative stomatitis, leukopenia, nausea and abdominal distress. Others reported are malaise, undue fatigue, chills and fever, dizziness and decreased resistance to infection. Incidence and severity of side effects are generally considered dose-related. *Skin:* erythematous rashes, pruritus, urticaria, photosensitivity, depigmentation, alopecia, ecchymosis, telangiectasia, acne, furunculosis. Concomitant exposure to ultraviolet radiation may aggravate lesions of psoriasis. *Blood:* bone marrow depression, leukopenia, thrombocytopenia, anemia, hypogammaglobulinemia, hemorrhage from various sites, septicemia. *Alimentary System:* gingivitis, pharyngitis, stomatitis, anorexia, vomiting, diarrhea, hematemesis, melena, gastrointestinal ulceration and bleeding, enteritis, hepatic toxicity resulting in acute liver atrophy, necrosis, fatty metamorphosis, periportal fibrosis, or hepatic cirrhosis. *Urogenital System:* renal failure, azotemia, cystitis, hematuria, defective oogenesis or spermatogenesis, transient oligospermia, menstrual dysfunction, infertility, abortion, fetal defects, severe nephropathy. *Pulmonary System:* Interstitial Pneumonitis. Deaths have been reported; chronic interstitial obstructure pulmonary disease has occasionally occurred. *CNS:* headaches, drowsiness, blurred vision. Aphasia, hemiparesis, paresis and convulsions have also occurred. Leucoencephalopathy following intravenous administration to patients who have had craniospinal irradiation has been reported. CNS toxicity which may occur after intrathecal use can be classified as follows: 1) chemical arachnoiditis manifested by such symptoms as headache, back pain, nuchal rigidity, and fever; 2)paresis, usually transient, manifested by paraplegia associated with involvement with one or more spinal nerve roots; 3) leucoencephalopathy manifested by confusion, irritability, somnolence, ataxia, dementia, and occasionally major convulsions.

Other reactions related or attributed to Methotrexate, such as metabolic changes, precipitating diabetes, osteoporotic effects, abnormal tissue cell changes and even sudden death have been reported.

ANTIDOTE FOR OVERDOSAGE Leucovorin (citrovorum factor) is a potent agent for neutralizing immediate toxic effects of Methotrexate on the hematopoietic system. Where large doses or overdoses are given, Calcium Leucovorin may be administered by intravenous infusion in doses up to 75 mg within 12 hours, followed by 12 mg intramuscularly every 6 hours for 4 doses. Where average doses of Methotrexate appear to have an adverse effect, 2 to 4 ml (6 to 12 mg) of Calcium Leucovorin may be given intramuscularly every 6 hours for 4 doses. In general, where overdosage is suspected, the dose of Leucovorin should be equal to or higher than the offending dose of Methotrexate and should best be administered within the first hour. Use of Calcium Leucovorin after an hour delay is much less effective.

CAUTION: Pharmacist: Because of its potential to cause severe toxicity, Methotrexate therapy requires close supervision of the patient by the physician. Pharmacists should dispense no more than a seven (7) day supply of the drug at one time. Refill of such prescriptions should be by direct order (written or oral) of the physician only.

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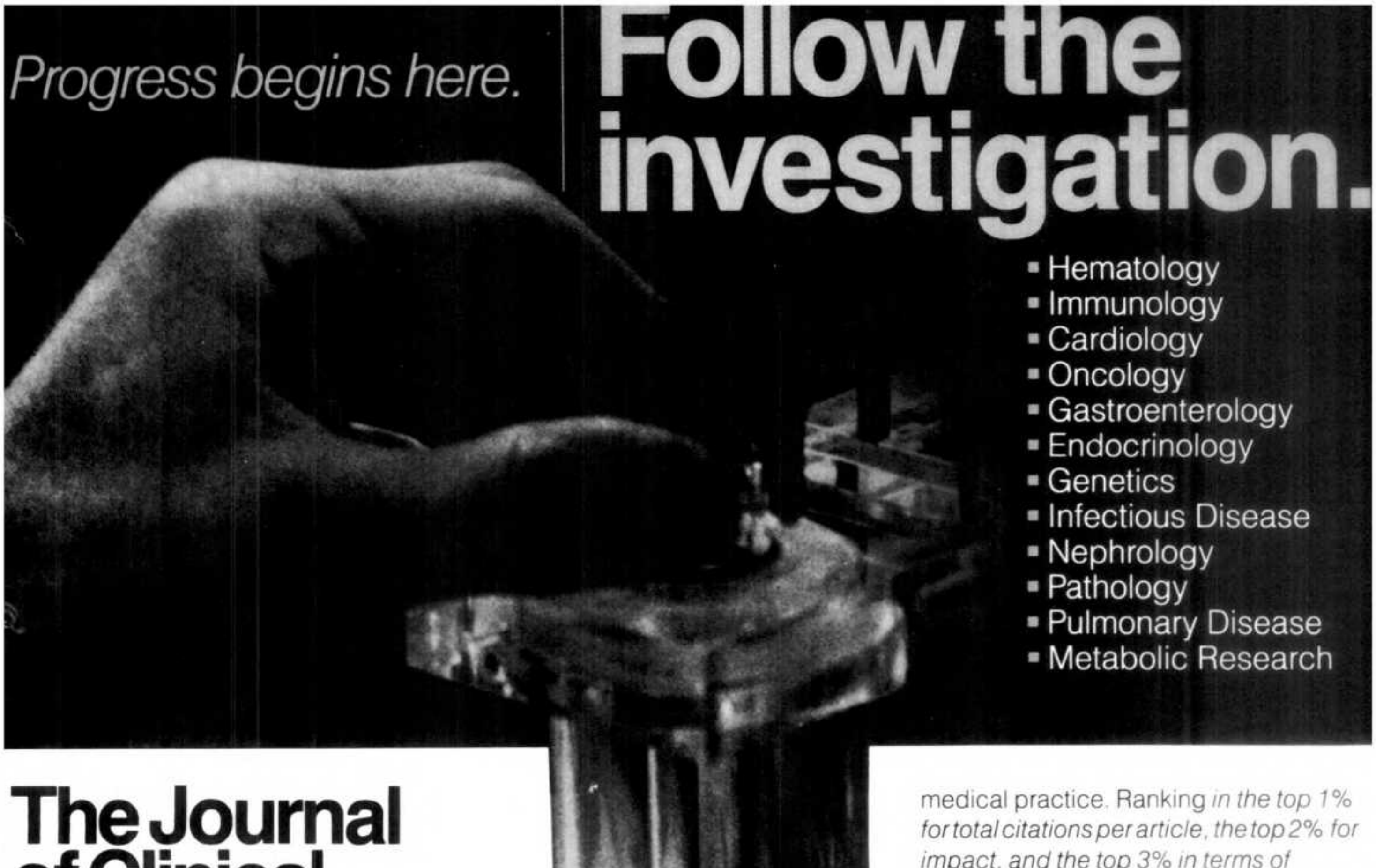

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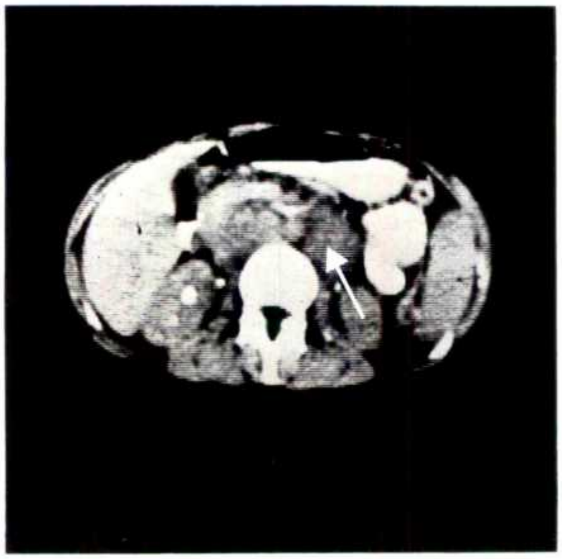
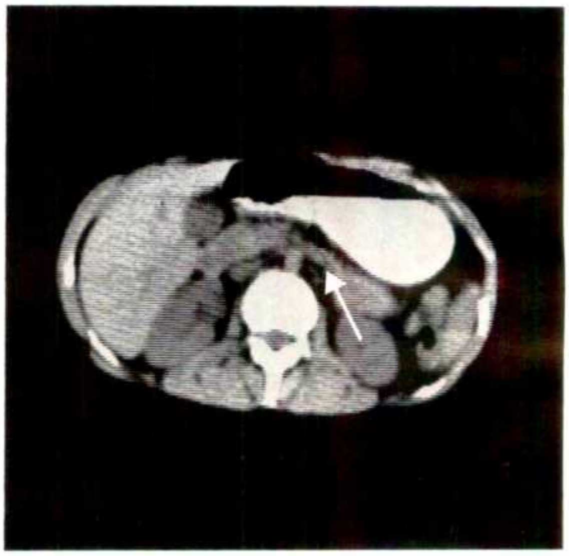


Massive retroperitoneal, retrocrural, para-aortic, and probable right iliac adenopathy.

Abdominal/Pelvic CT
August 4



No evidence of abnormal abdominal or pelvic adenopathy after Blenoxane combination chemotherapy.

CHOP-Bleo

1. Newcomer LN, Cadman EC, Nerenberg MI, *et al:* Randomized study comparing doxorubicin, cyclophosphamide, vincristine, methotrexate with leucovorin rescue, and cytarabine (ACOMLA) with cyclophosphamide, doxorubicin, vincristine, prednisone, and bleomycin (CHOP-B) in the treatment of diffuse histiocytic lymphoma. *Cancer Treat Rep* 66:1279-1284, 1982.

BACOP

2. Schein PS, DeVita VT Jr, Hubbard S, *et al:* Bleomycin, Adriamycin, cyclophosphamide, vincristine, and prednisone (BACOP) combination chemotherapy in the treatment of advanced diffuse histiocytic lymphoma. *Ann Intern Med* 85:417-422, 1976.

COP-BLAM

3. Laurence J, Coleman M, Allen SL, *et al:* Combination chemotherapy of advanced diffuse histiocytic lymphoma with the six-drug COP-BLAM regimen. *Ann Intern Med* 97:190-195, 1982.

*Case information on file at Bristol-Myers Oncology Division

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4. Skarin AT, Canellos GP, Rosenthal DS, *et al:* Improved prognosis of diffuse histiocytic and undifferentiated lymphoma by use of high dose methotrexate alternating with standard agents (M-BACOD). *J Clin Oncol* 1:91-98, 1983.
5. Anderson KC, Skarin AT, Rosenthal DS, *et al:* Combination chemotherapy for advanced non-Hodgkin's lymphomas other than diffuse histiocytic or undifferentiated histologies. *Cancer Treat Rep* 68:1343-1350, 1984.

m-BACOD

6. Skarin A, Canellos G, Rosenthal D, *et al:* Moderate dose methotrexate (m) combined with bleomycin (B), Adriamycin (A), cyclophosphamide (C), Oncovin (O) and dexamethasone (D), *m*-BACOD, in advanced diffuse histiocytic lymphoma (DHL). *Proc Am Soc Clin Oncol* 2:220, 1983.

MACOP-B

7. Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med* 102:596-602, 1985.

See prescribing information on following page.

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WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents.

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy. Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{co}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{co} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{co} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the postoperative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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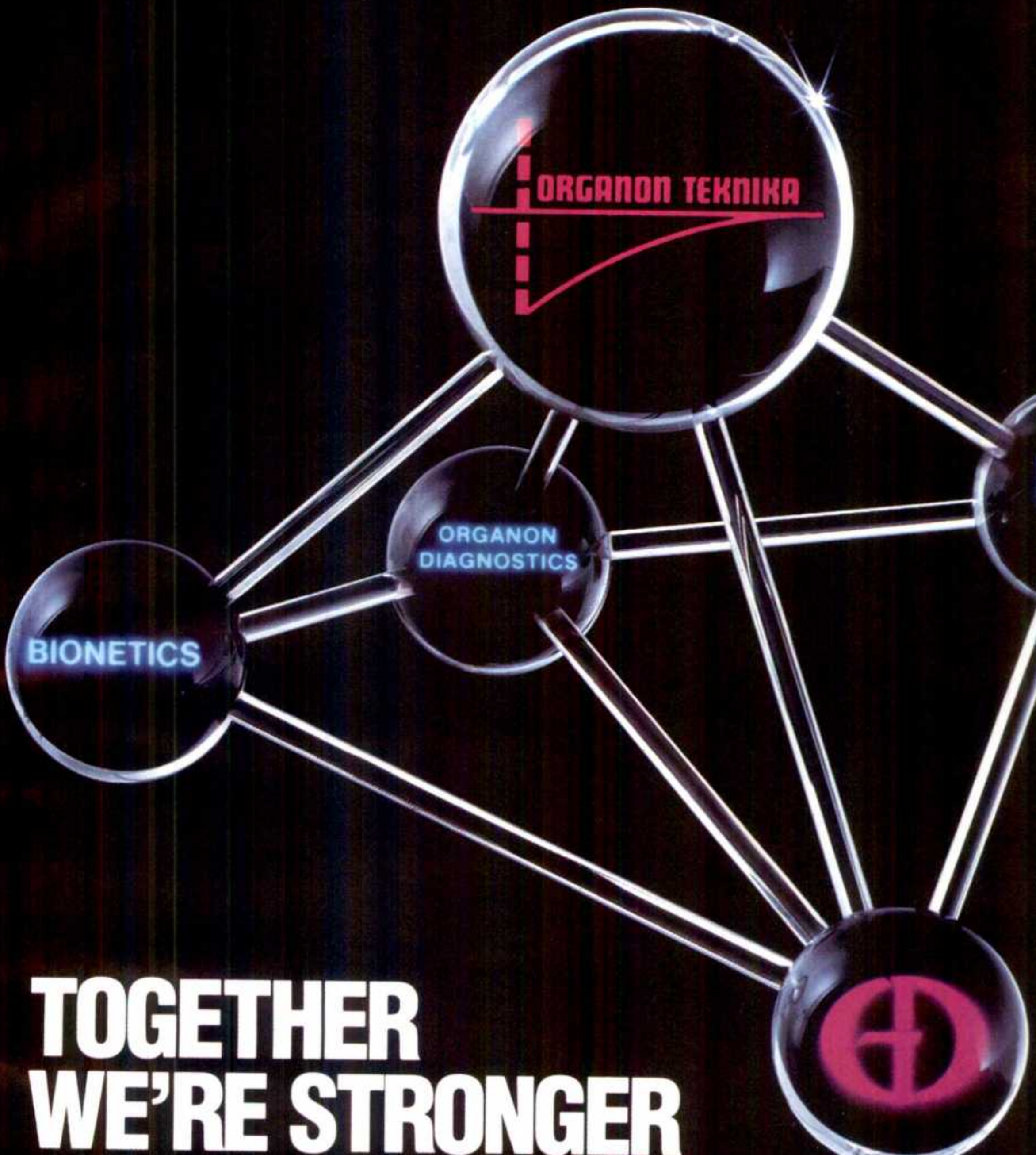

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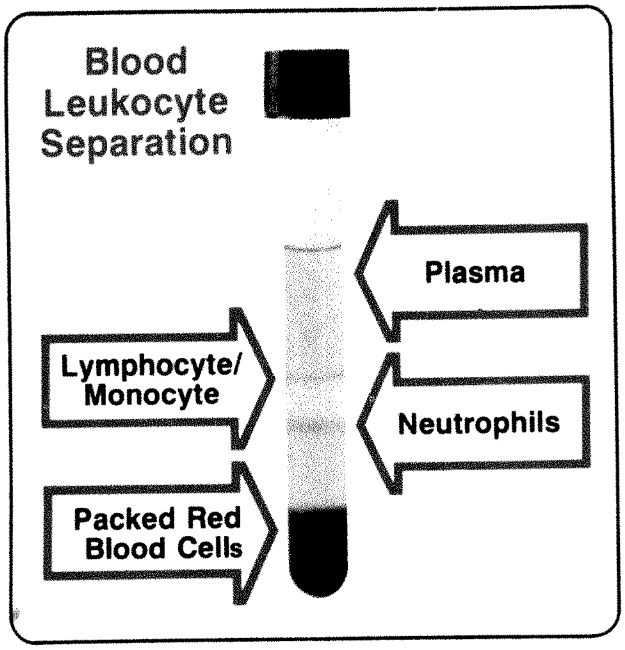

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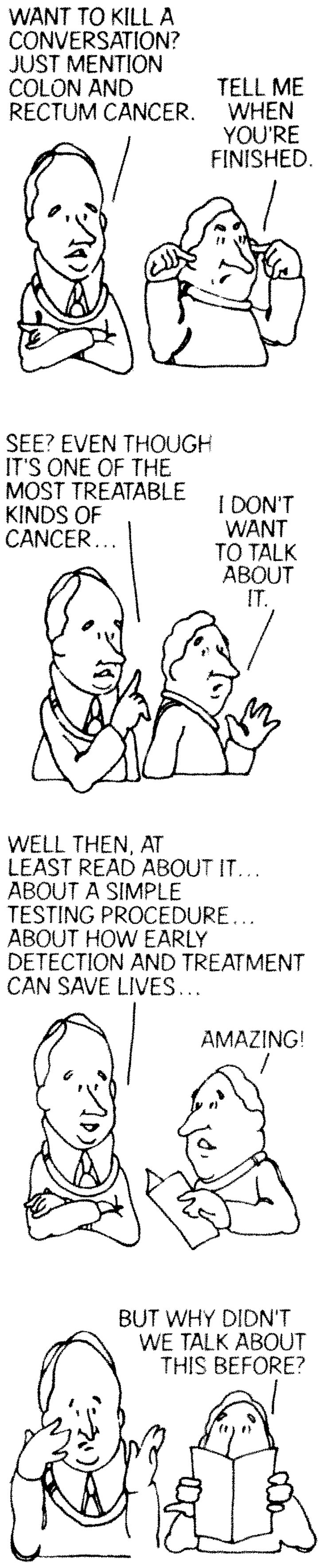
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Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: 1. Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

An effective response to a life-threatening infection
ZOVIRAX® I.V. INFUSION
(acyclovir sodium) sterile powder

An effective response to a life-threatening infection

ZOVIRAX® I.V. INFUSION
(acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug.

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days.

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: 1. C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. 33:1452-1463, 1973.

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85-ZOV-34

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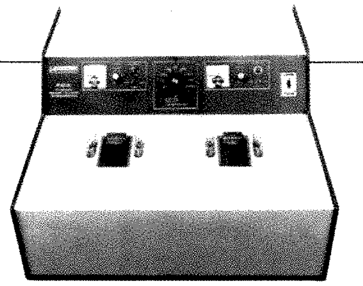

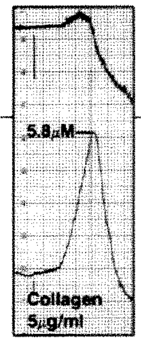

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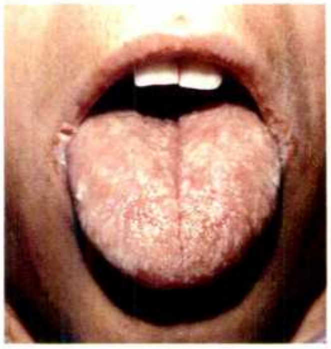
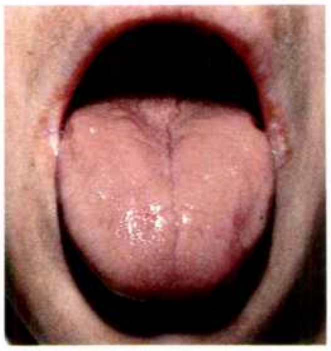
The first troche treatment for oropharyngeal candidiasis.



Before treatment.[1]



After treatment with MYCELEX Troche.[1]

High clinical cure* rates.

A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

Dramatic improvement in symptoms.

Patients attain a high degree of relief at the end of therapy, as evidenced by the dramatic decrease in severity of glossitis, patches, perleche, pain, and other clinical signs and symptoms of thrush.[4]

Good patient acceptance, minimal side effects.

With MYCELEX Troche, messy suspensions and lengthy swishing are things of the past. MYCELEX Troche has a pleasant, slightly sweet taste. And an entire day's therapy—5 troches—can easily be carried in pocket or purse.

Based on a study of 52 episodes of oropharyngeal candidiasis, the investigators stated: "We believe that the troche form of clotrimazole...in the treatment of thrush in any patient population...is both effective and safe."[2†]

*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

Mycelex® Troche

(clotrimazole) 10 mg

MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy. Pregnancy Category C. Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 095.

Store below 86°F (30°C), avoid freezing.

References: **1.** Montes LF, et al. Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals

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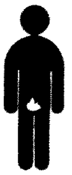
DOM-2242

SIX WARNING SIGNS OF KIDNEY RELATED DISEASES

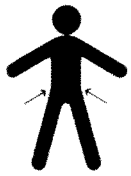


Burning or difficulty during urination

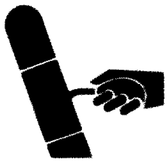
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



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Please see brief summary
of prescribing information
on next page.

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LEUCOVORIN CALCIUM

LEUCOVORIN CALCIUM INJECTION
Military Depot: NSN 6505-01-054-7008,
50 mg/vial for I.M. use

DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection. Preservative: benzyl alcohol 0.9% w/v. The inactive ingredients are sodium chloride 0.56% w/v. Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7.7.

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection. Contains no preservative. Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol. The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8.1. When reconstituted as directed, the resulting solution must be used within seven days. If the product is reconstituted with Water for Injection USP, use immediately.

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid.

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase. There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg.

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists. (See Warnings). (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy, and infancy when oral therapy is not feasible.

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient.

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive.

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

DOSAGE: Megaloblastic anemia: No more than or up to 1 mg daily. There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg; additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg.

For the treatment of overdosage of folic acid antagonists: To be given in amounts equal to the weight of the antagonist given.

HOW SUPPLIED:
NDC 0205-4004-51—6—1 ml Ampuls
NDC 0205-5330-92—50 mg Vial of Cryodesiccated powder

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LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information.

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS).

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS: *General:* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: Teratogenic Effects: Pregnancy Category C. Animal reproduction studies have not been conducted with leucovorin. It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Leucovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers. It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother.

Pediatric Use: (See Drug Interactions.)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[9,10,11]

REFERENCES:

1. Nixon PF and Bertino JR: Effective Absorption and Utilization of Oral Formyltetrahydrofolate in Man. *N Engl J Med 286*(4):175-179, Jan 1972.
2. Ratanasthien K, Blair JA, Leeming RJ, Cooke WT and Melikian V: Folates in Human Serum. *J Clin Pathol 27*:875-879, 1974.
3. Mehta BM, Gisolfi AL, Hutchison DJ, Nirenberg A, Kellick MG and Rosen G: Serum Distribution of Citrovorum Factor and 5-methyltetrahydrofolate Following Oral and I.M. Administration of Calcium Leucovorin in Normal Adults. *Cancer Treat Rep 62*(3):345-350, Mar 1978.
4. Data on file, Clinical Research, Lederle Laboratories, Pearl River, NY 10965.
5. Whitehead VM, Pratt R, Viallet A and Cooper BA: Intestinal Conversion of Folinic Acid to 5-methyltetrahydrofolate in Man. *Br J Haematol 22*:63-72, 1972.
6. Herbert V: Aseptic Addition for *Lactobacillus casei* Assay of Folate Activity in Human Serum. *J Clin Path 19*:12-16, 1966.
7. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer 41*(1):36-51, 1978.
8. Frei E, Blum RH, Pitman SW, *et al*: High Dose Methotrexate with Leucovorin Rescue: Rationale and Spectrum of Antitumor Activity. *Am J Med* 68:370-376, 1980.
9. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulphamethoxazole Inhibition of Haematopoiesis *in Vitro*. *Br J Haematol*, 40(3):363-367, 1978.
10. Steinberg SE, Campbell CL, Rabinovitch PS, *et al*: The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood*, 55(3):501-504, 1980.
11. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX. Toxoplasmosis. *J Infect Dis 135*(3):493-496, 1977.

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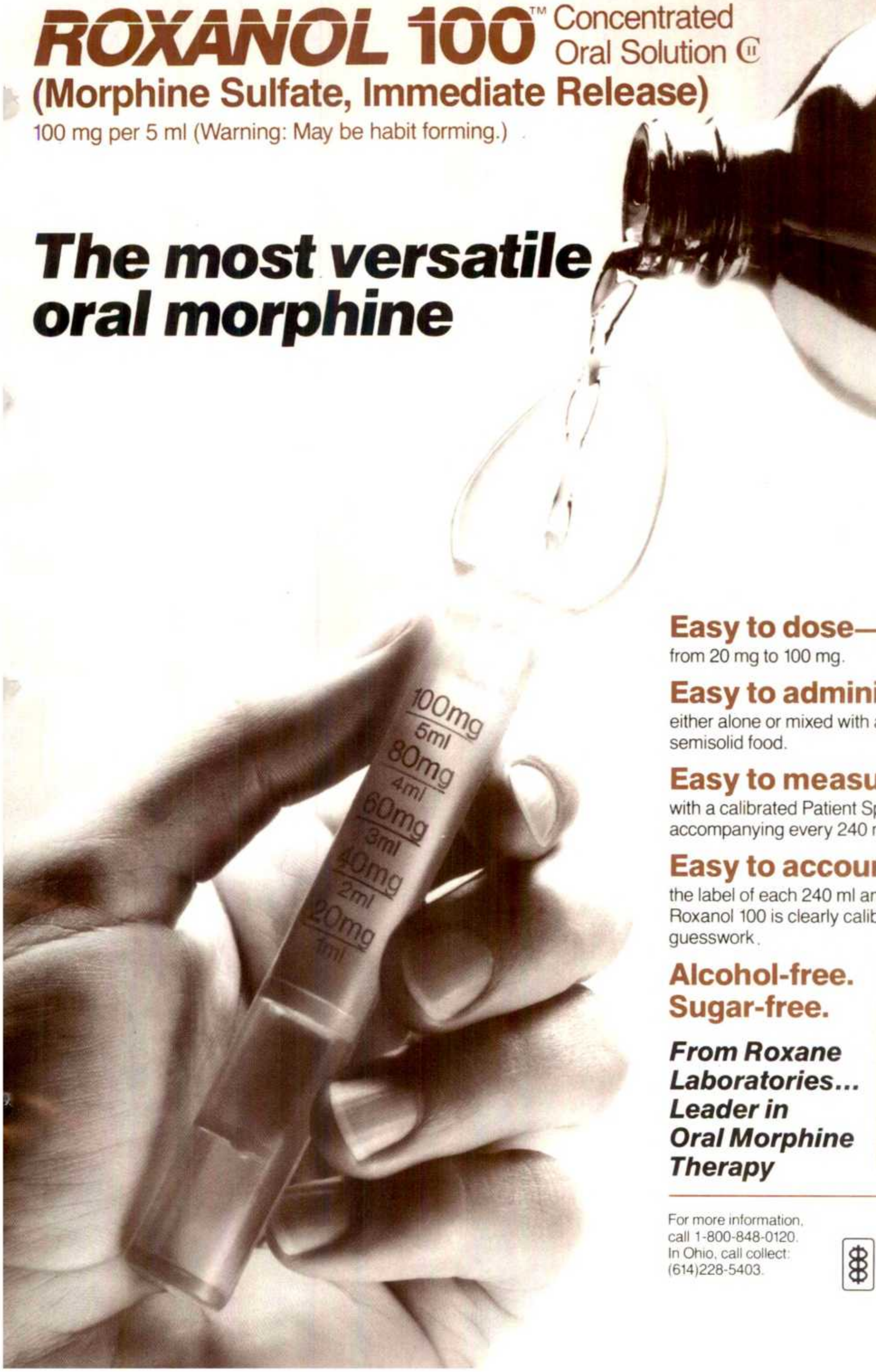

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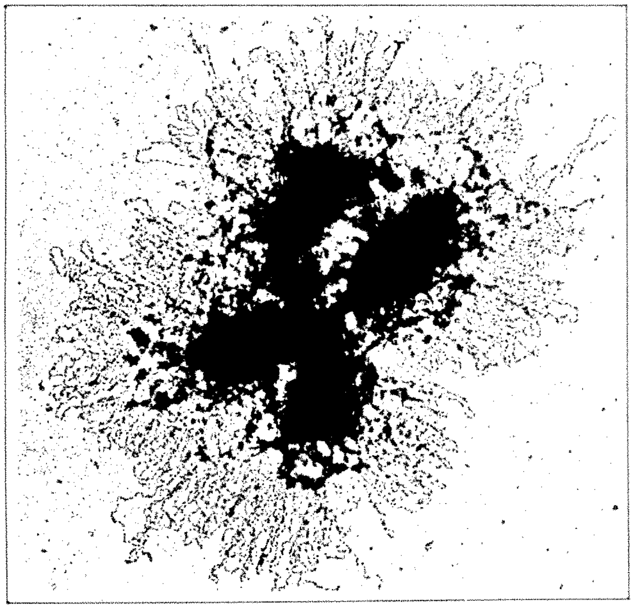

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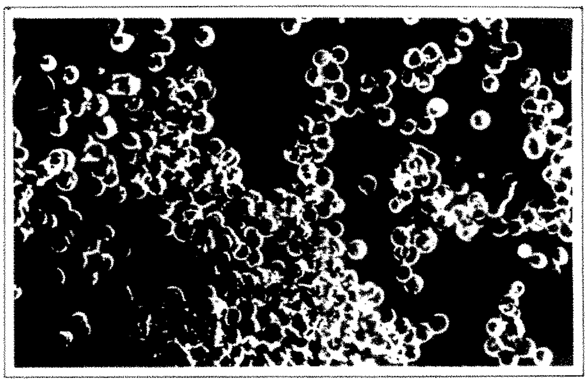

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REVIEW

The Structural and Functional Properties of Thrombospondin

By Jack Lawler

IN 1971 BAENZIGER and co-workers[1] identified a 190,000-dalton polypeptide that was selectively released from human platelets as a result of exposure to thrombin. The next ten years brought a gradual accumulation of information, mostly on the structure and subcellular location of this protein, designated thrombospondin, that has paved the way for a rapid expansion of information during the past four years. Recent advances include (1) the detection of thrombospondin in several cell types; (2) the concept of thrombospondin as a multifunctional protein with binding sites for heparin, calcium, fibrinogen, fibronectin, type V collagen, plasminogen, histidine-rich glycoprotein, and sulfated glycolipids; (3) the identification of potential functions for thrombospondin in cell physiology; and (4) a concept of native calcium-dependent structure. Thrombospondin can become associated with cell surfaces and has been shown to have lectin-like activity in hemagglutination assays. Collectively, these data have led to a concept of thrombospondin as an adhesive protein that is involved in cell-to-cell and cell-to-matrix interactions. Such interactions may be specifically involved in the association of many cell types with extracellular matrices. These interactions may be especially important at the surface of the platelet and at the site of vascular injury.

Thrombospondin has been identified by several names in the literature. Baenziger et al[1,2] designated the 190,000-dalton polypeptide as the thrombin-sensitive protein (TSP). When it was realized that TSP was actually a subunit of a larger protein, the name thrombospondin was proposed for the native protein to distinguish it from its subunits that, although equivalent in molecular weight (mol wt), may not be identical and to emphasize that the protein was secreted in response to thrombin, rather than released as a result of direct proteolysis.[3] Other investigators have chosen to emphasize the fact that thrombospondin is localized in the α-granules of platelets and have designated it glycoprotein Ig or glycoprotein G (GPG).[4-7] The glycoprotein characteristic is also emphasized in some of the literature on several types of cells in culture that identifies the subunit of thrombospondin as GP-160 or GP.[8,9]

MOLECULAR CHARACTERIZATION

Thrombospondin migrates as a single band of mol wt 142,000 to 190,000 during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent. The mol wt value obtained depends on the SDS-PAGE system used. In our hands,[10] thrombospondin migrates at 185,000 daltons on gels prepared by the method of Laemmli[11] and 145,000 daltons on phosphate-buffered gels prepared by the method of Weber and Osborn[12] (Fig 1). Sedimentation equilibrium analysis in the presence of β-mercaptoethanol and 6 mol/L guanidine-HCl gives a value of 113,000 to 138,000 daltons, and thus supports the lower SDS-PAGE values[13] (Table 1).

In the absence of reducing agents, thrombospondin migrates as a single band with an estimated mol wt of 450,000 (Fig 1).[3,14] Although, in general, mol wt determined by SDS-PAGE in the absence of reducing agents are suspect, this value agrees quite well with the value of 420,000 daltons obtained from sedimentation equilibrium analysis.[13] The ratio of the mole wt of the individual chains to the intact molecule, as determined by both sedimentation equilibrium and SDS-PAGE analysis, is approximately 1:3, indicating that the native thrombospondin molecule comprises three polypeptides of equivalent mol wt.[3,13] The three-chain structure is further supported by the observation that three bands with mol wt corresponding to one, two, and three chains are obtained by SDS-PAGE under conditions of partial reduction.[3] The chains of thrombospondin appear to be identical in terms of mol wt, NH_2-terminal amino acid sequence, and cleavage sites for thrombin, plasmin, thermolysin, trypsin, and chymotrypsin.[15-20] Isoelectric focusing data of Clemetson et al[21] indicate that the thrombospondin chains are heterogeneous in terms of charge, with isoelectric points ranging from pH 5.2 to 5.8. Isoelectric heterogeneity of the

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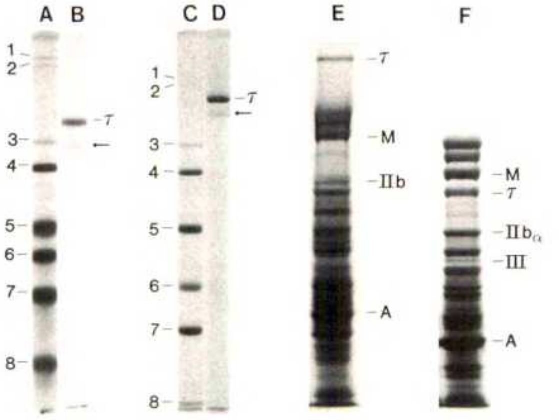

Fig 1. SDS-PAGE of thrombospondin on 5% Weber-Osborn phosphate-buffered gels (A, B), 7.5% Laemmli gels (C, D), and 3.5% to 10% Laemmli gradient gels (E, F). Gels A and C are a mixture of standard proteins composed of (1) spectrin α-subunit (240,000), (2) spectrin β-subunit (220,000), (3) β-galactosidase (130,000), (4) phosphoralase a (94,000), (5) bovine serum albumin (68,000), (6) catalase (56,000), (7) creatine kinase (40,000), and (8) carbonic anhydrase (29,000). Gels B and D are purified thrombospondin (τ) and a 160,000-dalton breakdown product of thrombospondin (arrow). Gels E and F are washed platelets that were solubilized in Laemmli sample buffer prepared without (E) and with (F) dithiothreitol. The electrophoretic mobility of myosin (M), thrombospondin (τ), glycoprotein IIb (IIb) and its α-subunit (IIbα), glycoprotein III (III), and actin (A) are indicated.

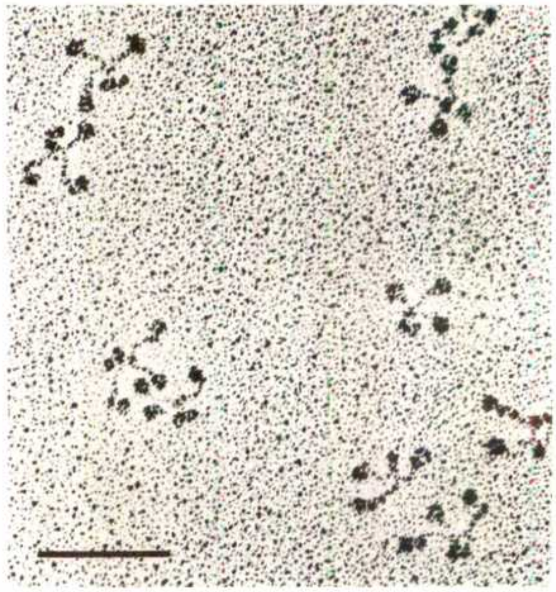

Fig 2. Electron microscopy of thrombospondin. Thrombospondin (100 μL) that was isolated in the presence of 2 mmol/L $CaCl_2$ was mixed with 900 μL of 70% glycerol, 0.15 mol/L ammonium acetate, and 200 μmol/L $CaCl_2$ just before spraying on freshly cleaved mica. The samples were rotary shadowed with platinum from an angle of 4.5 degrees to the horizontal,[16] *bar* = 100 nm.

chains of thrombospondin produced by human umbilical endothelial cells has also been described by Mosher et al.[9] Whether this charge heterogeneity arises from differences in primary sequence or from differences in posttranscriptional modification is unknown.

Recent immunologic, biochemical, and electron microscopic data permit the formulation of models for the structure of human platelet thrombospondin.[16-18] These models assume that the three chains of thrombospondin have similar conformation. Electron microscopy of replicas produced by low-angle rotary shadowing indicate that the thrombospondin molecule can be divided into four distinct structural regions: (1) globular region N, (2) the region in which the chains are cross-linked, (3) the thin connecting region, and (4) the globular region C (Figs 2 and 3).[16,18] Several pieces of evidence indicate that globular region N comprises the NH_2-terminal portions from each of the three chains of thrombospondin. A monoclonal antibody that is directed against a 30,000-dalton NH_2-terminal proteolytic fragment

Table 1. Structural Properties of Thrombospondin

Molecular weight	420,000
Molecular weight after reduction	
Laemmli SDS-PAGE	185,000
Weber-Osborn SD-PAGE*	145,000
Sedimentation equilibrium	133,000
Sedimentation coefficient†	9.7S
Intrinsic viscosity†	21 mL/g
Axial ratio of prolate ellipsoid model	3.3
Extinction coefficient‡	10.5–11.3
Secondary structure	
α-helix	11%
β-sheet	43%
random coil	46%

Molecular weight was determined by sedimentation equilibrium in 20 mmol/L Tris-HCl (pH 7.6), 0.15 mol/L NaCl, and 1 mmol/L EDTA.

*Electrophoresed in phosphate buffer.

†Determined in 15 mmol/L Tris-HCl (pH 7.6), 0.14 mol/L NaCl, 0.02% NaN_3, and 2 mmol/L $CaCl_2$.

‡Extinction of a 1% solution in a 1-cm path length cuvette at 280 nm and neutral pH.

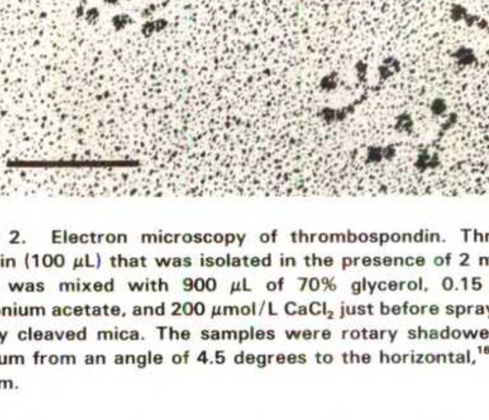
globular region C
globular region N
S S n
COOH
NH2
NH2
COOH
heparin-binding region
type V collagen-binding region
calcium-sensitive structure
fibrinogen-binding region

Fig 3. Schematic model of the structural and functional organizaton of the thrombospondin molecule. The molecule is depicted as being composed of three chains with globular regions at each terminus. The open box represents the site of a yet-to-be determined number (n) of interchain disulfide bonds. In the lower part of the figure, the alignment of some of the binding regions is presented.

of thrombospondin binds specifically to globular region N.[16,18] The selective removal of this fragment by thrombin digestion in the presence of calcium results in the loss of globular region N.[16] In addition, globular region N can be resolved into three separate smaller structures in some electron micrographic images (Fig 2).[16,18]

The second discernible region in this model is the location where the subunits of thrombospondin are cross-linked (Fig 3). This region is closer to globular region N than it is to C and appears, by electron microscopy, to be the only site where the chains are cross-linked. Thrombospondin has been reported to contain 114 disulfide bonds, which are distributed throughout the molecule.[15] The electron microscopic data suggest that the interchain disulfide bonds are confined to a relatively small region and that the majority of disulfide bonds are intrachain.

The third structural region is the thin, connecting region (Figs 2 and 3). In rotary shadowed images, these regions adopt a variety of orientations.[16-18] In the presence of calcium the region has been reported to be 160 to 291 Å in length.[16,18] The length of the thin, connecting regions increases to 383 Å when the samples are treated with EDTA before electron microscopy.

The final distinct structural region of thrombospondin is globular region C (Figs 2 and 3). This region has been reported to be 118 to 170 Å in diameter and appears at the ends of each of the thin, connecting regions.[16,18] In the presence of EDTA, globular region C decreases in size, concomitant to the increase in the length of the thin, connecting region, suggesting that there is a redistribution of mass from globular region C to the thin, connecting region.[16] These data, in conjunction with monoclonal antibody binding studies, indicate that, in the presence of calcium, globular region C comprises approximately 70,000 daltons at or near the C-terminal of each chain.[16,18]

THROMBOSPONDIN AS A MULTIFUNCTIONAL PROTEIN

Multifunctional proteins are defined by the following criteria: (1) the existence of more than one function on a single polypeptide chain and (2) the existence of distinct autonomous domains for the different functions.[22] The presence of functional domains can be demonstrated by the isolation of proteolytic fragments of the original polypeptide, which maintain each of the functional activities. Multifunctional proteins are particularly well suited to be structural elements of intercellular and extracellular matrices because of their ability to bind to several macromolecules. The identification of thrombospondin as a multifunctional protein is, at this point in time, inconclusive. Although thrombospondin does bind to heparin,[15,19,23] fibronectin,[24,25] fibrinogen,[25,26] plasminogen,[27] histidine-rich glycoprotein (HRGP),[28] type V collagen,[29] calcium,[30] and sulfated glycolipids,[31] it is not known if these binding activities reflect separate autonomous domains. Hypothesizing that thrombospondin is a multifunctional protein is, nevertheless, useful from an operational standpoint in that it dictates an experimental approach to the elucidation of structure–function relationships in the molecule.

The interaction of thrombospondin with heparin. When the supernatant from thrombin-treated platelets is applied to a column of Sepharose 4B, thrombospondin coelutes with platelet factor 4 in a high-mol-wt peak, presumably due to the association of platelet factor 4 with proteoglycan.[3,13,32] To separate thrombospondin from platelet factor 4, we have applied the high-mol-wt peak to an affinity column of heparin-Sepharose and have found that thrombospondin, as well as platelet factor 4, is retained by the column. Thrombospondin is eluted from the column by increasing the ionic strength with 0.45 mol/L NaCl. The existence of an autonomous functional domain for heparin can be demonstrated by the application of proteolytic fragments of thrombospondin to heparin-Sepharose affinity columns. When thrombospondin is exposed to thrombin, plasmin, or trypsin, polypeptides are released that bind to immobilized heparin with an affinity comparable to that of the native molecule.[15] The heparin-binding peptides produced with these enzymes separate into multiple closely spaced bands during SDS-PAGE (mol wt ~30,000) and isoelectric focusing (pI 4.8 to 6.8).[15] In contrast, thermolysin degradation produces a homogeneous heparin binding peptide of 25,000 daltons.[19]

The NH_2-terminal amino acid sequence of the heparin-binding peptide produced by plasmin, thermolysin, or chymotryptic digestion is identical to the NH_2-terminal amino acid sequence of the intact chains of thrombospondin.[17-20] These data are consistent with enzymatic digestion data, which indicates that the heparin-binding domain is located at one of the terminals of the thrombospondin chains and that each chain contains a heparin-binding domain.[16] In the presence of calcium, the release of the heparin-binding peptides by thrombin is associated with a decrease in the mol wt of the native chain from 185,000 to 160,000.[10,16] Densitometer scans of Coomassie blue stained gels indicate that all of the 185,000-dalton chains can be converted to 160,000- and 30,000-dalton fragments. The structural data described in the previous section indicate that the NH_2-terminal 30,000-dalton fragments that contain the heparin-binding sites are contained in globular region N (Fig 3).[16,18]

The interaction of thrombospondin with calcium. The sedimentation coefficient, intrinsic viscosity, circular dichroism, and electron microscopic appearance of thrombospondin are profoundly affected by the removal of divalent cations with EDTA.[10] The calcium-dependent changes in the conformation of thrombospondin result in a small change in the circular dichroism of thrombospondin and an increase in the susceptibility of the molecule to proteolysis. Binding curves can be constructed by quantitation of the extent of change in thrombospondin conformation as a function of calcium concentration.[30] When the circular dichroism of thrombospondin at 220 nm is measured as a function of free calcium concentration, a sigmoidal curve is obtained, indicating that the binding of calcium to thrombospondin is cooperative and that at least 12 calcium ions bind to thrombospondin with an average dissociation constant of 1.2×10^{-4} mol/L. Similar results are obtained when the peptide pattern produced by limited tryptic digestion of thrombospondin is titrated with calcium.[30] The average dissociation constant is 1.0×10^{-4} mol/L at 25 °C. However, because this method is only sensiof thrombospondin binds specifically to globular region N.[16,18] The selective removal of this fragment by thrombin digestion in the presence of calcium results in the loss of globular region N.[16] In addition, globular region N can be resolved into three separate smaller structures in some electron micrographic images (Fig 2).[16,18]

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The interaction of thrombospondin with heparin. When the supernatant from thrombin-treated platelets is applied to a column of Sepharose 4B, thrombospondin coelutes with platelet factor 4 in a high-mol-wt peak, presumably due to the association of platelet factor 4 with proteoglycan.[3,13,32] To separate thrombospondin from platelet factor 4, we have applied the high-mol-wt peak to an affinity column of heparin-Sepharose and have found that thrombospondin, as well as platelet factor 4, is retained by the column. Thrombospondin is eluted from the column by increasing the ionic strength with 0.45 mol/L NaCl. The existence of an autonomous functional domain for heparin can be demonstrated by the application of proteolytic fragments of thrombospondin to heparin-Sepharose affinity columns. When thrombospondin is exposed to thrombin, plasmin, or trypsin, polypeptides are released that bind to immobilized heparin with an affinity comparable to that of the native molecule.[15] The heparin-binding peptides produced with these enzymes separate into multiple closely spaced bands during SDS-PAGE (mol wt ~30,000) and isoelectric focusing (pI 4.8 to 6.8).[15] In contrast, thermolysin degradation produces a homogeneous heparin binding peptide of 25,000 daltons.[19]

The NH_2-terminal amino acid sequence of the heparin-binding peptide produced by plasmin, thermolysin, or chymotryptic digestion is identical to the NH_2-terminal amino acid sequence of the intact chains of thrombospondin.[17-20] These data are consistent with enzymatic digestion data, which indicates that the heparin-binding domain is located at one of the terminals of the thrombospondin chains and that each chain contains a heparin-binding domain.[16] In the presence of calcium, the release of the heparin-binding peptides by thrombin is associated with a decrease in the mol wt of the native chain from 185,000 to 160,000.[10,16] Densitometer scans of Coomassie blue stained gels indicate that all of the 185,000-dalton chains can be converted to 160,000- and 30,000-dalton fragments. The structural data described in the previous section indicate that the NH_2-terminal 30,000-dalton fragments that contain the heparin-binding sites are contained in globular region N (Fig 3).[16,18]

The interaction of thrombospondin with calcium. The sedimentation coefficient, intrinsic viscosity, circular dichroism, and electron microscopic appearance of thrombospondin are profoundly affected by the removal of divalent cations with EDTA.[10] The calcium-dependent changes in the conformation of thrombospondin result in a small change in the circular dichroism of thrombospondin and an increase in the susceptibility of the molecule to proteolysis. Binding curves can be constructed by quantitation of the extent of change in thrombospondin conformation as a function of calcium concentration.[30] When the circular dichroism of thrombospondin at 220 nm is measured as a function of free calcium concentration, a sigmoidal curve is obtained, indicating that the binding of calcium to thrombospondin is cooperative and that at least 12 calcium ions bind to thrombospondin with an average dissociation constant of 1.2×10^{-4} mol/L. Similar results are obtained when the peptide pattern produced by limited tryptic digestion of thrombospondin is titrated with calcium.[30] The average dissociation constant is 1.0×10^{-4} mol/L at 25 °C. However, because this method is only sensitic. Leung and Nachman[26] reported that the affinity of fluid-phase fibronectin for substrate-bound thrombospondin is considerably lower than that of fluid-phase fibrinogen. In contrast, Lahav et al[25] have reported that fluid-phase thrombospondin has virtually identical affinity for either substrate-bound fibronectin or fibrinogen. The most notable differences between the two studies are (1) calcium was either added or removed during purification of thrombospondin and (2) in one study, thrombospondin was in the fluid-phase, whereas in the other, it was substrate-bound. Both the presence of calcium and the interaction with the plastic surface could alter the conformation of thrombospondin in such a way that its binding properties might vary.

The interaction of thrombospondin with collagen. In an effort to begin characterizing the molecular associations that may be important to the formation of the extracellular matrix, Mumby et al[29] have examined the interaction of thrombospondin with various types of collagens. Thrombospondin was found to bind to type V collagen that was adsorbed to microtiter plates or cross-linked to Sepharose. In contrast, these authors reported that thrombospondin did not bind to type I, III, or IV collagen. Affinity chromatography of a chymotryptic digest of thrombospondin on type V collagen-Sepharose indicated that a fragment of thrombospondin, with a mol wt of 70,000 after reduction, contained the collagen-binding domain. The 70,000-dalton collagen-binding fragment was shown to be distinct from the heparin-binding site that was contained in a 28,000-dalton chymotryptic fragment.[29] This fragment is derived from the thin, connecting region and the region in which the chains are cross-linked by disulfide bonds (Fig 3).[18,49]

The interaction of thrombospondin with HRGP and plasminogen. Some recent studies describe additional binding data obtained using solid-phase binding assays.[27-29] Fluid-phase HRGP has been reported to bind to solid-phase thrombospondin with an apparent dissociation constant of 6.2 nmol/L.[28] Complex formation between thrombospondin and HRGP was also observed with both proteins in solution by sucrose density gradient ultracentrifugation. It is difficult to assess the physiologic significance of such a complex because the function of both proteins is only partially understood. Purified HRGP retards fibrinolysis by a mixture of tissue plasminogen activator and plasminogen.[50] In related experiments, solid-phase thrombospondin has also been shown to bind to fluid-phase plasminogen with an apparent dissociation of 35 nmol/L.[27] The interaction of thrombospondin with plasminogen is inhibited by ϵ-amino-*n*-caproic acid, suggesting that the lysine-binding sites of plasminogen are involved.[27] A trimolecular complex of thrombospondin, HRGP, and plasminogen has been shown to form.[51] The trimolecular complex was capable of binding to heparin and was an effective substrate for tissue plasminogen activator.[51] By binding to HRGP and plasminogen, thrombospondin may serve to regulate proteolysis by plasmin. These interactions are probably most significant at the platelet surface, where the thrombospondin concentration would be expected to be the highest.

The interaction of thrombospondin with thrombin. Exposure of thrombospondin to α-thrombin in the presence of calcium results in the selective removal of a 30,000-dalton heparin-binding fragment from the NH_2-terminal of each chain.[10,15,16,52] Additional sites for α-thrombin proteolysis are exposed when calcium is removed from the molecule with EDTA.[16,52] Cleavages at these sites result in the production of fragments of 130,000, 95,000, 65,000, and 25,000 daltons.[16,52] These fragments are produced, at least in part, by removal of mass from the COOH-terminal.[16] Takahashi et al[52] have reported that thrombospondin is not a substrate for γ-thrombin.

A complex of thrombospondin and radiolabeled thrombin has recently been identified.[53,54] Crossed immunoelectrophoresis of the proteins released from platelets that had been incubated with ^{125}I-thrombin revealed the radiolabeling of immunoprecipitates representing thrombospondin, albumin, and the "line" form of platelet factor 4.[53] Danishefsky et al[54] have identified the mechanism for the formation of the thrombospondin–thrombin complex.[54] They report that thrombospondin contains a free thiol group that is capable of reacting with a disulfide bond of thrombin. This thiol–disulfide exchange results in the formation of a covalent bond between thrombospondin and thrombin. This complex forms within 30 minutes after the addition of ^{125}I-α-thrombin to washed platelets and is found associated with the platelets and in the supernatant from the thrombin-treated platelets.[55]

Additional thrombospondin interactions. Fluid-phase thrombospondin also binds to solid-phase laminin, von Willebrand factor, factors IXa and Xa, and low-density lipoprotein.[29] These interactions should be viewed as tentative because they were not corroborated by a second type of binding assay. In a related study, thrombospondin has been shown to be substrate for factors IXa and Xa.[20]

The specific binding of thrombospondin to galactosylceramide-I^3-sulfate (sulfatide) has been demonstrated by probing thin-layer chromatograms of erythrocyte and platelet lipids with radiolabeled thrombospondin.[31] The binding of thrombospondin to sulfatide could be inhibited by disulfide bond reduction of thrombospondin, heparin, and a monoclonal antibody directed against the heparin-binding region (Segment I, Fig 3) of thrombospondin.[18,31] These data suggest that the interaction of thrombospondin with sulfatides may form the basis for the hemagglutinating activity of thrombospondin (see later).

THE OCCURRENCE OF THROMBOSPONDIN IN TISSUES

Platelets and megakaryocytes. The recent development of radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) for thrombospondin have made it possible to quantitate thrombospondin in various tissues.[9,44,45,56-60] Dawes et al[56] and Switalska et al[60] have reported that the thrombospondin content of platelets is 89.1 ± 28.3 ng/10^6 platelets and 67 ± 2.6 ng/10^6 platelets, respectively. A somewhat lower value of 24.6 ng/10^6 platelets has been reported by Ginsberg et al.[58] McLaren[61] has demonstrated the presence of thrombospondin in megakaryocytes by immunofluorescent staining, suggesting that platelet thrombospondin is synthesized in the megakaryocyte and not taken up by the platelet during circulation. Furthermore, the synthesis of metabolically labeled thrombospondin by megakaryocytes in culture has recently been reported.[62] Subcellular fractionation studies by several groups revealed that thrombospondin was associated with a granule population that sedimented to 1.16 to 2.1 g/cm^3 on sucrose density gradients.[3,63-65] These granules have been identified as α-granules on the basis of morphology and protein markers such as platelet factor 4, β-thromboglobulin–related antigen, and platelet fibrinogen. Recently, thrombospondin has been shown to be an α-granule constituent by crossed immunoelectrophoresis[23] and by transmission electron microscopy of antithrombospondin-stained ultrathin frozen sections.[66] Additional evidence that thrombospondin is an α-granule component comes from the study of patients with gray platelet syndrome.[4,58,67] Gray platelet syndrome is a rare inherited platelet disorder characterized by the absence of α-granules (see later). Thrombospondin, in addition to other α-granule proteins, is deficient in these patients.[4,58,67]

Exposure of platelets to thrombin results in the rapid secretion of α-granule constituents. Thus, although the level of thrombospondin circulating in plasma is low (97 to 163 ng/mL), the concentration of thrombospondin in serum increases to 15.3 to 17.5 μg/mL.[44,56,57,60] From comparisons of whole blood and serum levels of thrombospondin, Dawes et al[57] report that approximately 50% of the total thrombospondin is associated with the clot. These data are consistent with the observations that thrombospondin can bind to fibrinogen and platelets (see later).

Endothelial cells. Cultured endothelial cells synthesize and secrete a high-mol-wt glycoprotein, designated GP160 or GP, which is (1) metabolically labeled with radioactive amino acids or sugars, (2) resistant to digestion with bacterial collagenase, and (3) distinct from fibronectin.[68,69] This glycoprotein has been isolated from human and bovine endothelial cells and compared with human and bovine platelet thrombospondin, respectively.[8,9] McPherson et al[8] found that GP160 is similar to platelet thrombospondin on the basis of amino acid composition, electrophoretic mobility in SDS-PAGE, and two-dimensional peptide mapping by high-voltage electrophoresis followed by thin-layer chromatography. In addition, anti-GP160 antibodies cross-react with platelet thrombospondin.[8] Mosher et al[9] have shown that human endothelial cell GP160 and platelet thrombospondin have similar properties on two-dimensional nonreduced/reduced SDS-PAGE and two-dimensional isoelectric focusing/SDS-PAGE. These authors also showed that GP160 could be selectively immunoisolated from postculture medium with an antithrombospondin antibody.

Mosher et al[9] and Hunter et al[70] have reported that confluent human umbilical vein endothelial cells secrete 49 ± 4 μg/10^6 cells per 24 hours and 8.7 ± 1.7 μg/10^6 cells per 24 hours, respectively. By contrast, bovine aortic endothelial cells have been reported to secrete 1 μg/10^6 cells per 18 hours.[44] Whereas endothelial cells derived from human umbilical vein and human saphenous vein have comparable levels of intracellular thrombospondin, the cells derived from umbilical vein had a fivefold greater rate of thrombospondin secretion than those derived from the saphenous vein.[70] The subcellular localization of thrombospondin within the endothelial cell is distinct from that of von Willebrand protein.[71] The production of thrombospondin by bovine aortic endothelial cells, human vascular smooth muscle cells, and human skin and embryonic lung fibroblasts has been reported to be inversely proportional to the cell density.[72] By contrast, the amount of fibronectin and collagen produced by human skin fibroblasts is not affected by cell density.[72] These data suggest that production of thrombospondin is regulated by different factors than those that regulate fibronectin and collagen biosynthesis.

Kramer et al[73] have recently studied the extracellular matrix proteins, including thrombospondin, produced by human microvascular endothelial cells isolated from neonatal and adult skin. They found that although the secretion of thrombospondin is comparable for the two cell types, the amount that is incorporated into the matrix is much greater in the neonatal cell cultures.[73] Negligible staining reaction with antithrombospondin antibodies is observed with the matrix produced by endothelial cells from adult skin.[73] In contrast, Wight et al[74] have reported staining of the small vessels within the dermis with antithrombospondin antibodies; however, the source of the skin tissue was not specified in their study.

Other cell types. The observation that endothelial cells synthesize thrombospondin stimulated the search for thrombospondin in other cell types. Thrombospondin has now been identified in human and rat aortic smooth muscle cells,[44,48] human foreskin and fetal lung fibroblasts,[44,45] rat alveolar type II epithelial cells,[75] human monocytes,[76,77] and mouse peritoneal macrophages.[76] The observation of thrombospondin in rat alveolar tissue represents the first report that epithelial cells synthesize and secrete thrombospondin. Raugi et al[44] and Jaffe et al[45] have used immunofluorescent techniques to study the distribution of thrombospondin in cultures of endothelial cells, smooth muscle cells, and fibroblasts. Besides the fibrillar staining pattern described in the previous section, Jaffe et al[45] observed a punctate immunofluorescent staining pattern that disappeared when the cell layer was extracted with 1% deoxycholate. Both groups observed brightly fluorescent granules when the cells were permeabilized before staining. This punctate fluorescent pattern has been identified as thrombospondin that is contained in endocytic vesicles and is destined for degradation.[47] McKeown-Longo et al[47] have suggested that the presence of thrombospondin in endocytic vesicles, as well as its rapid turnover, indicates that the degradation of thrombospondin by human fibroblasts occurs by a receptor-mediated endocytosis.

The production of thrombospondin concomitant to fibronectin in endothelial cells and fibroblasts prompted Jaffe et al[76] to determine whether thrombospondin was also synthesized and secreted in concert with fibronectin by monocytes. They identified a protein that could be metabolically labeled with ^{35}S-methionine and was immunologically and structurally similar to thrombospondin in the culture medium of human monocytes and murine peritoneal macrophages.[76] The rate of thrombospondin synthesis by human monocytes was found to be 1.45 μg/10^6 cells per 24 hours.[76] In the murine system, thrombospondin synthesis was specifically depressed during macrophage activation.[76] Schwartz et al[77] have reported that synthesis of thrombospondin by human peripheral blood monocytes is decreased in the presence of lipopolysaccharide in a lymphocyte-independent manner.

Proteins, which are similar to thrombospondin but were not specifically identified as thrombospondin, are also synthesized and secreted by HT-1080 sarcoma cells and hamster embryo fibroblasts.[78,79] The production of fibronectin and the protein that is similar to thrombospondin by the hamster embryo fibroblasts could not be detected after the cells were transformed with polyoma virus.[78] However, in SV40-transformed fibroblasts, the production of thrombospondin did not decrease concomitant to the decrease in fibronectin production.[72]

The localization of thrombospondin in several human tissues has recently been described.[74] Thrombospondin was found to be present in the peritubular connective tissue of kidney tubules, at the base of glandular epithelia within the skin and lungs, at the dermal–epidermal junction of the skin, and partially surrounding skeletal muscle fibers.[74] With the exception of the Bowman's capsule and the glomeruli of the kidney, the localization of thrombospondin is similar to basement membrane components.[74] These data agree well with the cell culture data and support the hypothesis that thrombospondin is a constituent of the extracellular matrix.

THE ROLE OF THROMBOSPONDIN IN PLATELET FUNCTION

Although the specific role of thrombospondin in platelet function is unclear, the hypothesis that thrombospondin is involved in platelet aggregation is supported by the observations that (1) secreted thrombospondin can become associated with the platelet surface, (2) thrombospondin may be the endogenous platelet lectin, and (3) antithrombospondin antibodies inhibit thrombin-induced platelet aggregation.

Association of thrombospondin with the platelet surface. Several lines of evidence indicate that a portion of the thrombospondin that is released from the α-granules during platelet activation becomes associated with the platelet surface. In 1972, Majerus and Brodie[80] observed a twofold increase in the apparent number of binding sites for Lens culinaris lectin on the surface of platelets after thrombin treatment. Subsequent electron microscopic studies indicated that thrombin caused a decrease in the number of Lens culinaris receptor sites on the external platelet surface and an increase in the number of sites within the center of the canalicular system.[81] Feagler et al[81] proposed that this increase in receptor sites may be associated with the fusion of α-granule membranes with the plasma membrane. McGregor et al[82] have shown that Lens culinaris binds to thrombospondin as well as to glycoproteins Ib, IIb, IIIa, and IIIb. The possibility that receptors for thrombospondin appear on the platelet surface as a result of α-granule membrane fusion is supported by the crossed immunoelectrophoresis studies of Gogstad et al.[83] These authors homogenized the α-granules and separated the membranes from the soluble fraction. In addition to the thrombospondin in the soluble fraction, which is presumably released into the extracellular phase after platelet activation, a significant amount of thrombospondin was selectively associated with the α-granule membranes.

The presence of thrombospondin on the platelet surface after thrombin treatment has been demonstrated by immunologic binding studies and surface labeling experiments.[5,6,84,85] Thrombospondin also becomes one of the principal surface proteins labeled by ^{125}I-diazotized diiodosulfanilic acid or lactoperoxidase-catalyzed iodination after platelets are treated with thrombin.[5,6] Phillips et al[5] demonstrated that the quantity of thrombospondin associated with the platelet surface dramatically increased in the presence of calcium. Furthermore, if calcium was added to platelets that were activated in the presence of EDTA, thrombospondin became associated with the platelets. The association of thrombospondin to the platelets was closely paralleled by the appearance of fibrinogen (or fibrin) on the platelet surface. Because thrombospondin has been shown to bind to fibrinogen, it is possible that the calcium-dependent association of thrombospondin with the platelet surface is mediated by the binding of thrombospondin to fibrinogen, which, in turn, binds to the calcium-dependent glycoprotein IIb and IIIa complex (Fig 4).[25,26] Using double-label immunoelectron microscopy, Asch et al[86] have demonstrated a specific coclustering of thrombospondin and the glycoprotein IIb/IIIa complex, as well as thrombospondin and fibrinogen, on the surface of stimulated platelets. Based on these and other data, it has been proposed that thrombospondin may function to reinforce the macromolecular complex of fibrinogen, glycoprotein IIb, and glycoprotein IIIa during aggregation (see later). Monoclonal antibodies that react with the glycoprotein IIb/IIIa complex have been shown to inhibit the association of thrombospondin with thrombin-treated platelets.[87] However, in the absence of fibrinogen, thrombospondin did not bind to purified glycoprotein IIb/IIIa complex in a solid-phase binding assay.[26]

It is difficult to determine the quantity of thrombospondin that might have bound to the platelet in the absence of calcium and gone undetected by the method of Phillips et al.[5] Radiolabeling of thrombospondin on the surface of platelets treated with thrombin in the presence of EDTA can be demonstrated.[6] Using immunofluorescent labeling and a hemagglutination assay for the presence of thrombospondin (see later), Gartner and Dockter[88] have detected thrombospondin on the surface of platelets stimulated with the ionophore A23187 in the absence of free calcium. Hourdille et al[84] have recently reported the immunocytochemical localization of thrombospondin on platelets that were treated with thrombin in the presence or absence of calcium. In the presence of calcium, thrombospondin molecules were detected in clusters on the platelet surface. In the presence of EDTA, the number and size of these clusters was diminished,

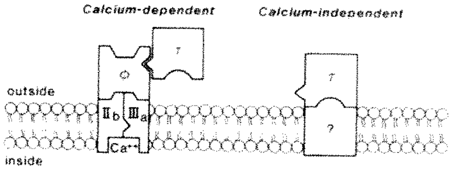

Fig 4. Schematic representations of the association of thrombospondin (τ) with the platelet membrane. The calcium-dependent mechanism is mediated by fibrinogen (φ) that binds to the glycoprotein IIb–IIIa complex that is formed in the presence of calcium. The calcium-independent mechanism would presumably involve a yet-to-be-determined receptor.

but some thrombospondin was still present on the platelet surface. Furthermore, the distribution of thrombospondin on thrombin-treated thrombasthenic platelets in either the presence or absence of calcium was similar to that observed for normal platelets treated with thrombin in the presence of EDTA.[84] These data indicate that there are two mechanisms for the association of thrombospondin with the platelet surface: a calcium-dependent mechanism, which may involve fibrinogen, and a calcium-independent mechanism, which may involve an, as yet, unidentified receptor (Fig 4).

Thrombospondin as the endogenous platelet lectin. Gartner et al[89] have shown that thrombin-activated human platelets express a membrane-bound lectin activity. Here, lectin activity is defined as the ability to agglutinate trypsinized, formalin-fixed erythrocytes. This activity is not present on the surface of unstimulated platelets, but is expressed after disruption or exposure to α-thrombin, γ-thrombin, thrombocytin, or the calcium ionophore A23187.[39] The endogenous lectin activity has also been reported to require 2 mmol/L calcium ions and 2 mmol/L magnesium ions for optimal expression and is inhibited by glucosamine, manosamine, arginine, and heparin.[36,40] Expression of the lectin activity was shown to be dependent on the release of platelet α-granules.[90] Jaffe et al[38] have shown that the purified thrombospondin can substitute for fixed (formaldehyde-treated) thrombin-treated platelets as the source of hemagglutination activity. These authors also showed that excess exogenous thrombospondin (or fibrinogen) could inhibit the hemagglutination activity of fixed thrombin-treated platelets, presumably by blocking thrombospondin receptors. The inhibitory activity of fibrinogen has been attributed to the fact that fibrinogen is the reputed receptor for endogenous platelet lectin activity.[38,39] The identification of thrombospondin as the endogenous lectin of platelets is consistent with the observation that antithrombospondin antiserum inhibits the lectin activity expressed by activated platelets or purified thrombospondin.[91,92] Biochemical and immunologic techniques have been used to identify the portions of the thrombospondin molecule that participate in hemagglutination.[35,91,92] The results indicate that multiple sites at or near the NH_2-terminal (Segment I) and COOH-terminal (Segment IV) may be involved.[18,35,91,92] Booth et al[93] have reported that only thrombospondin that has aggregated to form high-mol-wt multimers has hemagglutination activity. These aggregates are stabilized by intermolecular disulfide bonds.[93]

The role of thrombospondin in platelet aggregation. The role of thrombospondin in thrombin-, collagen-, adenosine diphosphate (ADP), and A23187-induced platelet aggregation has been studied using preparations of polyclonal or monoclonal antithrombospondin antibodies.[94-98] Thrombin-, collagen-, and A23187-induced platelet aggregation were inhibited by the prior addition of antithrombospondin antibodies.[94,95,97,98] The presence of the antibody preparations did not inhibit the release of dense granules or α-granules as measured by secretion of serotonin or β-thromboglobulin, respectively.[94,95,97] Whereas the primary phase of ADP-induced aggregation was not affected by the presence of antithrombospondin antibodies, the secretion-dependent secondary phase was completely inhibited.[94-96] The inhibition of aggregation probably involves perturbation of the thrombospondin–fibrinogen interaction in that (1) antithrombospondin Fab decreases the affinity of fibrinogen for its receptor and (2) antithrombospondin antibodies inhibit the ability of fixed platelets, bearing covalently bound fibrinogen, to interact with ADP-treated fresh platelets.[95,96] Leung[95] has proposed that thrombospondin stabilizes the fibrinogen–glycoprotein IIb/IIIa complex and in so doing affects the size and reversibility of platelet aggregates. The proposal is consistent with the observation that thrombospondin on the surface of thrombasthenic platelets does not support aggregation.[6,84] Thus, although the presence of thrombospondin seems to be a necessary condition for normal platelet aggregation, it is not a sufficient condition.

Based on the specificities of the antibody preparations, two groups have identified regions of the thrombospondin molecule that are involved in platelet aggregation.[94,97] Gartner et al[94] have found that antibodies directed against the NH_2-terminal heparin-binding fragment inhibit aggregation. Dixit et al[97] have reported that a monoclonal antibody against an 18,500-dalton chymotryptic fragment, which is derived from the COOH-terminal region of thrombospondin, inhibits aggregation.[18] These data suggest that multiple sites on the thrombospondin molecule are involved, with possibly one site binding to the platelet membrane and a second distinct site binding to the fibrinogen molecule.

DISEASE STATES OF THE PLATELET THAT AFFECT THROMBOSPONDIN

In 1971, Raccuglia[99] described a proband with poor clot retraction, prolonged bleeding time, and atypical platelet and megakaryocyte morphology. Light microscopy of the platelets from this individual revealed a dramatic diminution of granules and "a peculiar gray color," which lead to the identification of this disorder as gray platelet syndrome.[99] Subsequent morphological and biochemical studies indicate that such patients have a specific deficiency of platelet α-granules and a normal content of mitochondria, dense bodies, peroxisomes, and lysosomes.[4,67,100] Analysis of the protein composition of gray platelets revealed a decrease in the content of thrombospondin, fibrinogen, factor VIII-related antigen, fibronectin, albumin, platelet-derived growth factor, platelet factor 4, and β-thromboglobulin.[4,58,67] Functional studies indicate that gray platelets have decreased aggregation in response to ADP, thrombin, and collagen.[67] Gray platelets also have decreased expression of platelet-bound hemagglutination activity after treatment with thrombin and A23187.[90] Based on these data, Gerrard et al[67] have concluded that the α-granule proteins exert a potentiating effect on platelet aggregation in response to thrombin, collagen, and ADP. It is not possible to make any conclusions at this time about the specific role of thrombospondin because all of the α-granule proteins are apparently affected.

Essential thrombocythemia is a myeloproliferative disorder characterized by frequent bleeding and thrombotic complications.[101-109] The platelets from patients with this disorder have been reported to be functionally abnormal in that they (1) have decreased aggregation in response to thrombin, epinephrine, ADP, and collagen[101-104,108,109]; (2) have reduced activity of the platelet lipoxygenase pathway[105]; (3) are deficient in lipid peroxidation[106]; (4) have reduced serotonin uptake[101]; and (5) have decreased activation of platelet adenylate cyclase by prostaglandin D_2.[107] The platelets from these patients have some atypical morphology and can be enlarged.[101,104] Some of the functional and morphological defects have been reported to be corrected by treatment.[101,104]

On a molecular level, the platelets from patients with essential thrombocythemia have a variable decrease in the content of membrane glycoprotein I and an increase in the content of glycoprotein IV and a 170,000-dalton polypeptide.[7,108,109] Clezardin et al[110] have reported that all of the platelet membrane glycoproteins are significantly less sialylated in essential thrombocythemia, as compared with controls. The greatest decrease in labeling of terminal sialic acid is observed for GPIbα and GPIIIa.[110] Booth et al[7] have shown that the 170,000-dalton polypeptide is derived from thrombospondin. The electrophoretic pattern of nonreduced thrombospondin from these patients contained four closely spaced bands. A similar pattern is observed when thrombospondin is exposed to mild proteolysis and probably represents intact thrombospondin and thrombospondin molecules in which one, two, or three chains have been proteolyzed.[3] An increase in the 30,000-dalton, NH_2-terminal, heparin-binding peptide has been detected in concert with the increase in the high-mol-wt fragment.[111] These data suggest an increase in the susceptibility to proteolysis of a specific site in the thrombospondin molecule. Lens culinaris lectin binding studies indicate that there are carbohydrate moieties near this cleavage site and that the glycosylation of thrombospondin is abnormal in essential thrombocythemia.[110,111] Thus, defective glycosylation may lead to the increased proteolysis. Initial studies indicate that the abnormal proteolysis of thrombospondin also occurs in reactive thrombocytosis.[111] If these changes result from the defect in the rate of platelet production, then they, like some of the morphological and functional defects, may be ameliorated by treatment.

The plasma and urine levels of thrombospondin have been measured in several patient groups by RIA.[59,112] Increases in thrombospondin levels generally parallel increases in β-thromboglobulin and platelet factor 4.[59,112] Lane et al[112] concluded that β-thromboglobulin was the most sensitive marker of in vivo platelet release. However, under conditions of reduced clearance in patients with renal failure who are undergoing hemodialysis with heparin anticoagulation, thrombospondin may be the most reliable marker.[112] Thrombospondin levels have not been reported to be as sensitive to the presence of heparin as have platelet factor 4.[59,112] Harker et al[59] have reported a greater increase in thrombospondin than platelet factor 4 or β-thromboglobulin in patients undergoing thoracotomy. They suggest that some of this thrombospondin was derived from a non-platelet source.[59]

FUTURE DIRECTIONS

The data summarized here suggest that thrombospondin is a multifunctional protein with the ability to bind a broad spectrum of macromolecules. Through these various associations, thrombospondin may participate in cell-to-cell and cell-to-matrix interactions. In terms of its multifunctional character and its ability to bind to cell surfaces, thrombospondin is similar to fibronectin and laminin and should be considered as a member of a family of adhesive proteins.[113] Initial studies indicate that thrombospondin may have a specific role in the formation and assembly of the extracellular matrix and that thrombospondin biosynthesis is regulated by factors that are distinct from those that regulate other matrix components.[48,49,72-74] Majack et al[49] have reported that treatment of rat aortic smooth muscle cells in culture with platelet-derived growth factors results in the selective, dose-dependent synthesis of thrombospondin. Experiments on the effects of antithrombospondin antibodies and heparin indicated that thrombospondin is involved in smooth muscle cell migration.[114] In addition, vessels characterized by atherosclerosis exhibited marked immunofluorescent staining with antithrombospondin antibodies.[74] These observations represent an important first step toward an understanding of the role of thrombospondin in the pathobiology of atherosclerosis. In general, thrombospondin may function during cell migration in response to injury or during embryogenesis.

The developing fibrin clot probably represents a specialized example of an extracellular matrix. The incorporation of thrombospondin into fibrin and plasma clots can be demonstrated by determining the amount of thrombospondin that is associated with these clots and by immunofluorescent microscopy (Fig 5) (J. Lawler and D.K. Smith, unpublished observations, May 1983).[37,57,115,116] The immunofluorescent studies indicate that thrombospondin becomes uniformly incorporated into fibrin fibrils. Bale et al[115] have reported that most of the thrombospondin was incorporated into the

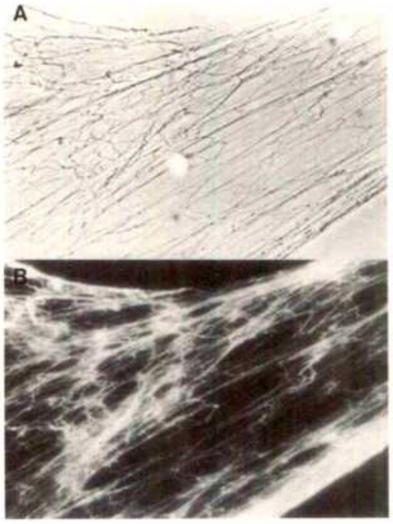

Fig 5. Immunofluorescent localization of thrombospondin in the fibrin clot. The platelets from 20 mL of platelet-rich plasma were pelleted and resuspended in 2.0 mL of Tris-buffered saline containing 2 mmol/L $CaCl_2$. The platelet suspension was treated with 0.10 U/mL of thrombin with mixing, and the aggregates were removed by centrifugation. The supernatant was mixed with 2.0 mL of platelet-poor plasma. The sample was treated with 0.01 U/mL of thrombin and was rapidly deposited on coverslips. After the clots formed, the coverslips were washed and stained with antithrombospondin antiserum, followed by fluorescein-conjugated goat antirabbit IgG. (A) Clot viewed under phase contrast. (B) The same field viewed by fluorescent microscopy.

clot while the fibrin was forming. In their study, thrombospondin was covalently incorporated into high-mol-wt complexes in the presence of factor XIIIa, suggesting that thrombospondin is a substrate for factor XIIIa.[115] In the presence of thrombospondin, the clots were composed of more numerous, thinner fibers than in the absence of thrombospondin.[115] The incorporation of thrombospondin into the fibrin clot may provide an additional structural component, may function to interface cell surfaces with the clot, or may regulate the fibrinolytic system through its interactions with HRGP and plasminogen.[27,28,51] Thus, besides a general function in fibrillogenesis, thrombospondin may have evolved with some specialized functions in the physiology of thrombosis.

Recent evidence indicates that activation of the platelet membrane results in the elaboration of specific sites for adhesion and aggregation.[84-87,95,117] The constituents of these sites seem to be a glycoprotein IIb/IIIa complex on the platelet membrane and a group of adhesive proteins, including fibrinogen, fibronectin, von Willebrand factor, and thrombospondin.[84-87,95,117] The specifics of the structure, composition, and assembly of these adhesion sites remain to be elucidated.

The view of thrombospondin as a multifunctional protein would predict that it is organized into discrete structural and functional regions. An important area for future work involves the determination of the arrangement of the functional activities of thrombospondin along each of the polypeptides. Limited proteolysis with a broad spectrum of proteases has been used to produce fragments of thrombospondin.[15-20,29,34-36] The 30,000-dalton heparin-binding peptides are readily released from the NH_2-terminal under a variety of conditions.[15-20] In addition, fragments of 70,000 daltons and 120,000 daltons have been reported to contain the collagen and fibrinogen binding sites, respectively.[29,36] The correlation of structure and function for the individual chains of thrombospondin should permit the development of a detailed model of the molecule. Such a model would provide a molecular basis for a better understanding of thrombospondin function in physiologic processes. Most of the thrombospondin interactions that have been described are based on in vitro assay systems that frequently involve macromolecules immobilized on plastic or Sepharose. These data must now be extended to more physiologic systems and more direct in vivo observations. Determination of the distribution of thrombospondin and its various ligands in tissues represents the first step toward establishing that these interactions have an opportunity to occur in vivo.[74]

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REFERENCES

1. Baenziger NL, Brodie GN, Majerus PW: A thrombin-sensitive protein of human platelet membranes. Proc Nat Acad Sci USA 68:240, 1971

2. Baenziger NL, Brodie GN, Majerus PW: Isolation and properties of a thrombin-sensitive protein of human platelets. J Biol Chem 247:2723, 1972

3. Lawler JW, Slayter HS, Coligan JE: Isolation and characterization of a high molecular weight glycoprotein from human blood platelets. J Biol Chem 253:8609, 1978

4. Nurden AT, Kunicki TS, Dupuis D, Soria C, Caen JP: Specific protein and glycoprotein deficiencies in platelets isolated from two patients with the gray platelet syndrome. Blood 59:709, 1982

5. Phillips DR, Jennings LK, Prasanna HR: Ca^{2+}-mediated association of glycoprotein G (thrombin-sensitive protein, thrombospondin) with human platelets. J Biol Chem 255:11629, 1980

6. George JN, Lyons RM, Morgan RK: Membrane changes associated with platelet activation. Exposure of actin on the platelet surface after thrombin-induced secretion. J Clin Invest 66:1, 1980

7. Booth WJ, Berndt MC, Castaldi PA: An altered platelet granule glycoprotein in patients with essential thrombocythemia. J Clin Invest 73:291, 1984

8. McPherson J, Sage H, Bornstein P: Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture: Apparent identity with platelet thrombospondin. J Biol Chem 256:11330, 1981

9. Mosher DF, Doyle MJ, Jaffe EA: Synthesis and secretion of thrombospondin by cultured human endothelial cells. J Cell Biol 93:343, 1982

10. Lawler J, Chao FC, Cohen CM: Evidence for calcium-sensitive structure in platelet thrombospondin: Isolation and partial characterization of thrombospondin in the presence of calcium. J Biol Chem 257:12257, 1982

11. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

12. Weber K, Osborn M: Proteins and sodium dodecyl sulfate: Molecular weight determination on polyacrylamide gels and related procedures, in Neurath H (ed): The Proteins, vol 1. Orlando, Fla, Academic Press, 1975, p 179

13. Margossian SS, Lawler JW, Slayter HS: Physical characterization of platelet thrombospondin. J Biol Chem 256:7495, 1981

14. Lawler JW, Chao FC, Fang PH: Observation of a high molecular weight platelet protein released by thrombin. Thromb Haemost 37:355, 1977

15. Lawler JW, Slayter HS: The release of heparin-binding peptides from platelet thrombospondin by proteolytic action of thrombin, plasmin and trypsin. Thromb Res 22:267, 1981

16. Lawler J, Derick LH, Connolly JE, Chen JH, Chao FC: The structure of human platelet thrombospondin. J Biol Chem 260:3762, 1985

17. Coligan JE, Slayter HS: Structure of thrombospondin. J Biol Chem 259:3944, 1984

18. Galvin NJ, Dixit VM, O'Rourke KM, Santoro SA, Grant GA, Frazier WA: Mapping of epitopes for monoclonal antibodies against human platelet thrombospondin with electron microscopy and high sensitivity amino acid sequencing. J Cell Biol 101:1434, 1985

19. Dixit VM, Grant GA, Santoro SA, Frazier WA: Isolation and characterization of a heparin-binding domain from the amino terminus of platelet thrombospondin. J Biol Chem 259:10100, 1984

20. Raugi GJ, Mumby SM, Ready CA, Bornstein P: Location and partial characterization of the heparin-binding fragment of platelet thrombospondin. Thromb Res 36:165, 1984

21. Clemetson KJ, Capitanio A, Pareti FL, McGregor JL, Luscher EF: Additional platelet membrane glycoprotein abnormali-

ties in Glanzmann's thrombasthenia: A comparison with normals by high resolution two-dimensional polyacrylamide gel electrophoresis. Thromb Res 18:797, 1980

22. Bisswanger H, Schmincke-Ott E: Multifunctional proteins. New York, Wiley, 1980

23. Gogstad GO, Solum NO, Krutnes M-B: Heparin-binding platelet proteins demonstrated by crossed affinity immunoelectrophoresis. Br J Haematol 53:563, 1983

24. Lahav J, Schwartz MA, Hynes RO: Analysis of platelet adhesion with a radioactive chemical crosslinking reagent: Interaction of thrombospondin with fibronectin and collagen. Cell 31:253, 1982

25. Lahav J, Lawler J, Gimbrone MA: Thrombospondin interactions with fibronectin and fibrinogen: Mutual inhibition in binding. Eur J Biochem 145:151, 1984

26. Leung LLK, Nachman RL: Complex formation of platelet thrombospondin with fibrinogen. J Clin Invest 70:542, 1982

27. Silverstein RL, Leung LLK, Harpel PC, Nachman RL: Complex formation of platelet thrombospondin with plasminogen. Modulation of activation by tissue activator. J Clin Invest 74:1625, 1984

28. Leung LLK, Nachman RL, Harpel PC: Complex formation of platelet thrombospondin with histidine-rich glycoprotein. J Clin Invest 73:5, 1984

29. Mumby SM, Raugi GJ, Bornstein P: Interactions of thrombospondin with extracellular matrix proteins: Selective binding to type V collagen. J Cell Biol 98:646, 1984

30. Lawler J, Simons ER: Cooperative binding of calcium to thrombospondin: The effect of calcium on the circular dichroism and limited tryptic digestion of thrombospondin. J Biol Chem 258:12098, 1983

31. Roberts DD, Havestick DM, Dixit VM, Frazier WA, Santoro SA, Ginsburg V: The platelet glycoprotein thrombospondin binds specifically to sulfated glycolipids. J Biol Chem 260:9405, 1985

32. Barber AJ, Kaser-Glanzmann R, Jakabova M, Luscher EF: Characterization of a chondroitin 4-sulfate proteoglycan carrier for heparin neutralizing activity (platelet factor 4) released from human blood platelets. Biochim Biophys Acta 286:312, 1972

33. Alexander RJ, Detwiler TC: Quantitative adsorption of platelet glycoprotein G (thrombin-sensitive protein, thrombospondin) to barium citrate. Biochem J 217:67, 1984

34. Haverstick DM, Dixit VM, Grant GA, Frazier WA, Santoro SA: Localization of the hemagglutinating activity of platelet thrombospondin to a 140,000-dalton thermolytic fragment. Biochemistry 23:5597, 1984

35. Haverstick DM, Dixit VM, Grant GA, Frazier WA, Santoro SA: Characterization of the platelet agglutinating activity of thrombospondin. Biochemistry 24:3128, 1985

36. Dixit VM, Grant GA, Frazier WA, Santoro SA: Isolation of the fibrinogen-binding region of platelet thrombospondin. Biochem Biophys Res Commun 119:1075, 1984

37. Tuszynski GP, Srivastava S, Switalska HI, Holt JC, Cierniewski CS, Niewiarowski S: The interaction of human platelet thrombospondin with fibrinogen. J Biol Chem 260:12240, 1985

38. Jaffe EA, Leung LLK, Nachman RL, Levin RI, Mosher DF: Thrombospondin is the endogenous lectin of human platelets. Nature 295:246, 1982

39. Gartner TK, Gerrard JM, White JG, Williams DC: Fibrinogen is the receptor for the endogenous lectin of human platelets. Nature 289:688, 1981

40. Gartner TK, Williams DC, Minion FC: Thrombin-induced platelet aggregation is mediated by a platelet plasma membrane-bound lectin. Science 200:1281, 1978

41. Zetter BR, Chen LB, Buchanan JM: Effects of protease treatment on growth, morphology, adhesion, and cell surface proteins of secondary chick embryo fibroblasts. Cell 7:407, 1976

42. Teng NNH, Chen LB: Thrombin-sensitive surface protein of cultured chick embryo cells. Nature 259:578, 1976

43. Lawler JW, Chen LB, Chao FC: Association of thrombospondin with the fibrillar network produced by chick embryo fibroblasts in culture. Fed Proc 41:1120, 1982 (abstr)

44. Raugi GJ, Mumby SM, Abbott-Brown D, Bornstein P: Thrombospondin: Synthesis and secretion by cells in culture. J Cell Biol 95:351, 1982

45. Jaffe EA, Ruggiero JT, Leung LLK, Doyle MJ, McKeown-Longo PJ, Mosher DF: Cultured human fibroblasts synthesize and secrete thrombospondin and incorporate it into extracellular matrix. Proc Natl Acad Sci USA 80:998, 1983

46. Hedman K, Johansson S, Vartio T, Kjellen L, Vaheri A, Hook M: Structure of the pericellular matrix: Association of heparan and chondroitin sulfates with fibronectin-procollagen fibers. Cell 28:663, 1982

47. McKeown-Longo PJ, Hanning R, Mosher DF: Binding and degradation of platelet thrombospondin by cultured fibroblasts. J Cell Biol 98:22, 1984

48. Majack RA, Cook SC, Bornstein P: Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. J Cell Biol 101:1059, 1985

49. Lawler J, Connolly J, Ferro P, Derrick LH: Thrombin and chymotrypsin interactions with thrombospondin in bioregulatory functions of thrombin, in Fenton JW II, Shuman D, Walz D (eds): Proc NY Acad Sci (in press)

50. Lijnen HR, Hoylaerts M, Collen D: Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. J Biol Chem 255:10214, 1980

51. Silverstein RL, Leung LLK, Harpel PC, Nachman RL: Platelet thrombospondin forms a trimolecular complex with plasminogen and histidine-rich glycoprotein. J Clin Invest 75:2065, 1985

52. Takahashi K, Aiken M, Fenton JW, Walz DA: Thrombospondin fragmentation by α-thrombin and resistance to γ-thrombin. Biochem J 224:673, 1984

53. Hagen I, Gogstad GO, Brosstad F, Solum NO: Demonstration of ^{125}I-labelled thrombin binding platelet proteins by use of crossed immunoelectrophoresis and autoradiography. Biochim Biophys Acta 732:600, 1983

54. Danishefsky KJ, Alexander RJ, Detwiler TC: Formation of a stable complex of thrombin and the secreted platelet protein glycoprotein G (thrombin-sensitive protein, thrombospondin) by thiol-disulfide exchange. Biochemistry 23:4984, 1984

55. Yeo K-T, Detwiler TC: Analysis of the fate of platelet-bound thrombin. Arch Biochem Biophys 236:399, 1985

56. Saglio SD, Slayter HS: Use of a radioimmunoassay to quantify thrombospondin. Blood 59:162, 1982

57. Dawes J, Clemetson KJ, Gogstad GO, McGregor J, Clezardin P, Prowse CV, Pepper DS: A radioimmunoassay for thrombospondin, used in a comparative study of thrombospondin, β-thromboglobulin and platelet factor 4 in healthy volunteers. Thromb Res 29:569, 1983

58. Ginsberg MH, Wencel JD, White JG, Plow EF: Binding of fibronectin to α-granule-deficient platelets. J Cell Biol 97:571, 1983

59. Harker LA, Marzec UM, Ginsberg MH: Thrombospondin levels in plasma, platelets and urine in normal subjects, subjects receiving heparin, thoracotomy patients, and patients undergoing cardiopulmonary bypass. Thromb Haemost 50:22, 1983 (abstr)

60. Switalska HI, Tuszynski GP, Cierniewski C, Niewiarowski S: Radioimmunoassay of human thrombospondin. Fed Proc 43:979, 1984 (abstr)

61. McLaren KM: Immunohistochemical localisation of thrombospondin in human megakaryocytes and platelets. J Clin Pathol 36:197, 1983

62. Rabellino EM, Awidi A, Sitar G, Levene RB: Human marrow megakaryocytes synthesize platelet glycoproteins IIb, IIIa and thrombospondin. Fed Proc 44:1126, 1985 (abstr)

63. Phillips DR, Agin PP: Thrombin substrates and the proteolytic site of thrombin action on human-platelet plasma membranes. Biochim Biophys Acta 352:218, 1974

64. Hagen I: Effects of thrombin on washed, human platelets: Changes in the subcellular fractions. Biochim Biophys Acta 392:242, 1975

65. Kaser-Glanzmann R, Jakabova M, Luscher EF: Subcellular localization of a glycoprotein released from human platelets upon stimulation by thrombin. Chimia 30:96, 1976

66. Wencel-Drake JD, Painter RG, Zimmerman TS, Ginsberg MH: Ultrastructural localization of human platelet thrombospondin, fibrinogen, fibronectin and von Willebrand factor in frozen thin section. Blood 65:929, 1985

67. Gerrard JM, Phillips DR, Rao GHR, Plow EF, Walz DA, Ross R, Harker LA, White JG: Biochemical studies of two patients with the gray platelet syndrome. J Clin Invest 66:102, 1980

68. Jaffe EA, Mosher DF: Synthesis of fibronectin by cultured human endothelial cells. J Exp Med 147:1779, 1978

69. Sage H, Crouch E, Bornstein P: Collagen synthesis by bovine aortic endothelial cells in culture. Biochemistry 18:5433, 1979

70. Hunter NR, Dawes J, MacGregor IR, Pepper DS: Quantitation by radioimmunoassay of thrombospondin synthesised and secreted by human endothelial cells. Thromb Haemost 52:288, 1984

71. Reinders JH, deGroot PG, Dawes J, Hunter NR, van Heugten HAA, Zandbergen J, Gonsalves MD, van Mourik JA: Comparison of secretion and subcellular localization of von Willebrand protein with that of thrombospondin and fibronectin in cultured human vascular endothelial cells. Biochim Biophys Acta 844:306, 1985

72. Mumby SM, Abbott-Brown D, Raugi GJ, Bornstein P: Regulation of thrombospondin secretion by cells in culture. J Cell Physiol 120:280, 1984

73. Kramer RH, Fuh GM, Bensch KG, Karasek MA: Synthesis of extracellular matrix glycoproteins by cultured microvascular endothelial cells isolated from the dermis of neonatal and adult skin. J Cell Physiol 123:1, 1985

74. Wight TN, Raugi GJ, Mumby SM, Bornstein P: Light microscopic immunolocation of thrombospondin in human tissues. J Histochem Cytochem 33:295, 1985

75. Sage H, Farin FM, Striker GE, Fisher AB: Granular pneumocytes in primary culture secrete several major components of the extracellular matrix. Biochemistry 22:2148, 1983

76. Jaffe EA, Ruggiero JT, Falcone DJ: Monocytes and macrophages synthesize and secrete thrombospondin. Blood 65:79, 1985

77. Schwartz BS, Doyle MJ, Westrick LL, Mosher DF: Human peripheral blood monocytes synthesize and secrete thrombospondin. Blood 62:87a, 1983 (abstr)

78. Carter WG, Hakomori S: A protease-resistant, transformation-sensitive membrane glycoprotein and an intermediate filament-forming protein of hamster embryo fibroblasts. J Biol Chem 253:2867, 1978

79. Alitalo K, Vaheri A, Krieg T, Timpl R: Biosynthesis of two subunits of type IV procollagen and of other basement membrane proteins by a human tumor cell line. Eur J Biochem 109:247, 1980

80. Majerus PW, Brodie GN: The binding of phytohemagglutinins to human platelet plasma membranes. J Biol Chem 247:4253, 1972

81. Feagler JR, Tillack TW, Chaplin DD, Majerus PW: The effects of thrombin on phytohemagglutinin receptor sites in human platelets. J Cell Biol 60:541, 1974

82. McGregor JL, Clemetson KJ, James E, Greenland T, Dechavanne M: Identification of human platelet glycoproteins in SDS-polyacrylamide gels using ^{125}I-labeled lectins. Thromb Res 16:825, 1979

83. Gogstad GO, Hagen I, Korsmo R, Solum NO: Evidence for release of soluble, but not of membrane-integrated proteins from human platelet α-granules. Biochim Biophys Acta 702:81, 1982

84. Hourdille P, Hasitz M, Belloc F, Nurden AT: Immunocytochemical study of the binding of fibrinogen and thrombospondin to ADP- and thrombin-stimulated human platelets. Blood 65:912, 1985

85. Cierniewski C, Switalska H, Hershock D, Capitanio AM, Tuszynski GP, Niewiarowski S: Association of α-granule proteins with platelet membranes and cytoskeletons. Fed Proc 43:659, 1984 (abstr)

86. Asch AS, Leung LLK, Polley MJ, Nachman RL: Platelet membrane topography: Colocalization of thrombospondin and fibrinogen with the glycoprotein IIb-IIIa complex. Blood 66:926, 1985

87. Plow EF, McEver RP, Coller BS, Woods VL, Marguerie GA, Ginsberg MH: Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. Blood 66:724, 1985

88. Gartner TK, Dockter ME: Secreted platelet thrombospondin binds monovalently to platelets and erythrocytes in the absence of free Ca^{2+}. Thromb Res 33:19, 1983

89. Gartner TK, Williams DC, Phillips DR: Platelet plasma membrane lectin activity. Biochem Biophys Res Commun 79:592, 1977

90. Gartner TK, Gerrard JM, White JG, Williams DC: The endogenous lectin of human platelets is an α granule component. Blood 58:153, 1981

91. Gartner TK, Doyle MJ, Mosher DF: Effect of anti-thrombospodin antibodies on the hemagglutination activities of the endogenous platelet lectin and thrombospondin. Thromb Haemost 52:354, 1984

92. Dixit VM, Haverstick DM, O'Rourke KM, Hennessey SW, Grant GA, Santoro SA, Frazier WA: Effects of anti-thrombospondin monoclonal antibodies on the agglutination of erythrocytes and fixed, activated platelets by purified thrombospondin. Biochemistry 24:4270, 1985

93. Booth WJ, Castaldi PA, Berndt MC: Platelet thrombospondin haemagglutinin activity is due to aggregate formation. Thromb Res 39:29, 1985

94. Gartner TK, Walz DA, Aiken M, Starr-Spires L, Ogilvie ML: Antibodies against a 23 kd heparin binding fragment of thrombospondin inhibit platelet aggregation. Biochem Biophys Res Commun 124:290, 1984

95. Leung LLK: Role of thrombospondin in platelet aggregation. J Clin Invest 74:1764, 1984

96. Agam G, Shohat O, Livne A: Thrombospondin plays a role in platelet-platelet recognition during release-related aggregation. Proc Soc Exp Biol Med 177:482, 1984

97. Dixit VM, Haverstick DM, O'Rourke KM, Hennessy SW, Grant GA, Santoro SA, Frazier WA: A monoclonal antibody against human thrombospondin inhibits platelet aggregation. Proc Natl Acad Sci USA 82:3472, 1985

98. Nurden AT, Hasitz M, Rosa J-P: Inhibition of thrombin-induced platelet aggregation by a rabbit antibody against human platelet thrombospondin. Thromb Haemost 50:132, 1983 (abstr)

99. Raccuglia G: Gray platelet syndrome: A variety of qualitative platelet disorder. Am J Med 51:818, 1971

100. White JG: Ultrastructural studies of the gray platelet syndrome. Am J Pathol 95:445, 1979

101. Spaet TH, Lejnieks I, Gaynor E, Goldstein ML: Defective platelets in essential thrombocythemia. Arch Intern Med 124:135, 1969

102. Ginsburg AD: Platelet function in patients with high platelet counts. Ann Intern Med 82:506, 1975

103. Weinfeld A, Branehog I, Kutti J: Platelets in the myeloproliferative syndrome. Clin Haematol 4:373, 1975

104. Castaldi PA, Berndt MC, Booth W, Gregory C, Bull H, Greaves M: Evidence for a platelet membrane defect in the myeloproliferative syndromes. Thromb Res 27:601, 1982

105. Schafer AI: Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. N Engl J Med 306:381, 1982

106. Keenan JP, Wharton J, Shepherd AJN, Bellingham AJ: Defective platelet lipid peroxidation in myeloproliferative disorders: A possible defect in prostaglandin synthesis. Br J Haematol 35:275, 1977

107. Cooper B, Schafer AI, Puchalsky D, Handin RI: Platelet resistance to prostaglandin D_2 in patients with myeloproliferative disorders. Blood 52:618, 1978

108. Bolin RB, Okumura T, Jamieson GA: Changes in distribution of platelet membrane glycoproteins in patients with myeloproliferative disorders. Am J Hematol 3:63, 1977

109. Eche N, Sie P, Caranobe C, Nouvel C, Pris J, Boneu B: Platelets in myeloproliferative disorders. III Glycoprotein profile in relation to platelet function and platelet density. Scand J Haematol 26:123, 1981

110. Clezardin P, McGregor JL, Dechavanne M, Clemetson KJ: Platelet membrane glycoprotein abnormalities in patients with myeloproliferative disorders and secondary thrombocytosis. Br J Haematol 60:331, 1985

111. Lawler J, Cohen AM, Chao FC, Moriarty DJ: Thrombospondin in essential thrombocythemia. Blood 67:555, 1986

112. Lane DA, Ireland H, Wolff S, Ranasinghe E, Dawes J: Detection of enhanced in vivo platelet α-granule release in different patient groups—comparison of β-thromboglobulin, platelet factor 4 and thrombospondin assays. Thromb Haemost 52:183, 1984

113. Yamada KM: Cell surface interactions with extracellular materials. Annu Rev Biochem 52:761, 1983

114. Majack RA, Cook SC, Bornstein P: Thrombospondin, a PDGF-inducible protein, may play a role in the regulation of vascular smooth muscle cell migration by glycosaminoglycans. Fed Proc 44:738, 1985 (abstr)

115. Bale MD, Westrick LG, Mosher DF: Incorporation of thrombospondin into fibrin clots. J Biol Chem 260:7502, 1985

116. Murphy-Ullrich JE, Mosher DF: Localization of thrombospondin in clots formed *in situ*. Fed Proc 43:704, 1984 (abstr)

117. Plow EF, Srouji AH, Meyer D, Marguerie G, Ginsberg MH: Evidence that three adhesive proteins interact with a common recognition site on activated platelets. J Biol Chem 259:5388, 1984

The *Dpg* Gene: An Intracorpuscular Modifer of Red Cell Metabolism

By Nancy A. Noble and Gerald Rothstein

The genetic locus designated *Dpg* has two alleles in outbred Long-Evans rats. Genotype at this locus affects quantities of red cell 2,3- diphosphoglycerate (DPG) and adenosine triphosphate, as well as activities of two important glycolytic enzymes: phosphofructokinase and pyruvate kinase. Intravascular red cell survival is shortened in low-DPG animals. In order to get closer to the specific action of this locus, we addressed the question of whether the *Dpg* gene acts through intracorpuscular or extracorpuscular factors. Bone marrow transplantation after total body irradiation and ^{51}Cr red cell survival after cross transfusion were the methods used. Because the animals that were used differed in hemoglobin phenotype, donor and recipient cells could be quantified in cross-transplanted animals. Phenotypic markers of *Dpg* genotype were measured in animals 40 to 50 days after transplantation. Values for these markers correlated highly with the percentage of donor and recipient cells present. In vivo survival of low-DPG red cells was significantly shorter than that of high-DPG cells ($P < .05$), regardless of the genotype of the recipient. From the present studies, we conclude that the action of the *Dpg* gene is exerted by an intracorpuscular factor.

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GENETICALLY DETERMINED VARIATIONS in the intra-erythrocytic quantity of 2,3-diphosphoglycerate (DPG) have been ascribed to the influence of a major locus, *Dpg*.[1] Outbred Long-Evans rat populations possess two alleles at this locus, *D* and *d*, with gene frequencies of 0.75 and 0.25, respectively (unpublished observation, July 1985).[2] This locus is about two recombination units away from β-globin.[2] Animals with genotypes *DD* or *Dd* possess high-DPG levels and are designated "H." Those with genotype *dd* have low-DPG levels and are designated "L." Although the *Dpg* locus was initially named only for its effect on DPG concentrations, it is now clear that this locus is associated with a variety of other alterations in red cell glycolysis and intravascular survival. For example, when compared with the H genotype, the red cells of L rats have approximately 40% lower activities of phosphofructokinase (PFK) and pyruvate kinase (PK) and 40% lower adenosine triphosphate (ATP) and DPG levels.[1,2,3] Although the precise mechanisms have not been identified, determinations of steady state glycolytic intermediate levels suggest that a relative block at the PFK step is responsible for lower glycolytic flux and lower steady-state levels of DPG and ATP in L red cells.[4] The lower in vitro activity of PK in these cells is thought to have no metabolic consequences. Recent work in this laboratory indicates that reticulocytes of the three genotypes have similar enzyme activities and metabolite levels, but that as these cells mature, *Dpg* phenotype is expressed.[5] In addition, the potential red cell life span, as measured by length of time for complete decay of ^{51}Cr-labeled red cells, is about 16% shorter for L cells than for H cells.[6]

Levels of DPG and ATP and activities of PFK and PK are considered the phenotypic markers of genotype at the *Dpg* locus. Although it is possible that *Dpg* represents a cluster of genes all affecting red cell metabolism, studies to date suggest that these effects are produced by one locus. Many commercially obtained Long-Evans rats have been tested, and all have either high or low values for all of these markers.[2] Also, congenic lines of rats, differing only in the area of *Dpg*, have recently been developed, and their behavior is consistent with the pleitropic effects of one locus (G.J. Brewer, University of Michigan, personal communication, April 1985).

There is considerable interest in understanding how a single genetic locus or closely linked genes can produce these changes, and the mechanism of action of *Dpg* can be generally divided into two categories: (1) extracorpuscular factors and (2) intracorpuscular factors that can influence glycolysis and cellular survival. If a distinction between these can be made, then further investigations as to the nature of the *Dpg* gene can be pursued more efficiently by considering only intracorpuscular or extracorpuscular causes, but not both.

In the present study, we have used bone marrow transplant and cross-transfusion techniques to establish that the action of the *Dpg* gene is dependent on an intracorpuscular factor or factors.

MATERIALS AND METHODS

Bone marrow transplantation. Experimental animals were of two genotypes: *AAdd* and *BBDD*. The *AA* and *BB* refer to genotypes and the $^{III}\beta$-globin locus, which is closely linked to the *Dpg* locus.[2] Three days before transplantation, 5- to 7-week-old male Long-Evans rats were given drinking water containing 40 mg/L gentamycin, 250 mg/L neomycin, and 1 g/L cefoxitin. One day before transplant, animals received 9.28 Gy (5.8 Gy/min) total body irradiation from a rotating platform in a Gammator containing a cesium source (Isomedix, Parsippany, NJ). Then, animals were housed singly in sterile cages and given sterile food, as well as water containing antibiotics.

Irradiated animals were given 4 to 6 × 10^6 bone marrow cells flushed from the tibias and femurs of single donors into Hanks' balanced saline solution. After collection, the cells were counted electronically, suspended in a volume of 0.6 to 0.9 mL per rat, and immediately injected into the tail vein.

Assay of erythrocyte constituents. All reagents were obtained from Sigma Chemical Co., St. Louis. Blood for enzyme assays was passed through a syringe-barrel column of alpha-cellulose:Sigmacell for removal of leukocytes and platelets.[7] Then, the washed packed cells were subjected to lysis in a 1:15 dilution of water, followed in one minute by the addition of an equal volume of the buffer containing 20 mmol/L KPi, 3 mm $MgSO_4$, 1 mmol/L fructose-6-phosphate, 5 mmol/L dithiothreitol, 100 μmol/L EDTA,

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and 20% (wt/vol) glycerol, pH 7.6.[8] Enzymatic assays were carried out according to Beutler,[7] and reactions were started with 0.05 mL of hemolysate. Results were expressed as IU/g hemoglobin. Hemolysate hemoglobin was measured by adding 0.02 mL hemolysate to 1 mL of Drabkin's reagent and measuring the absorbance at 540 nm. Concentrations of DPG and ATP were measured on perchloric acid extracts of whole blood using the Sigma Chemical Co. kits No. 35-UV and 366-UV, respectively. Red cell and white cell counts were measured electronically, using a ZBI Coulter counter (Coulter Electronics, Hialeah, Fla). Reticulocyte counts were determined by counting at least 1,000 cells on smears stained with new methylene blue.

Red cell survival. Forty-eight male rats 3 months of age were used. Half of these rats were of genotype *AADD* and half were *BBdd*. Note that these animals were in the opposite linkage phase from those used for bone marrow transplant. Twelve animals of each genotype were given ^{51}Cr-labeled red cells from the other 12 animals of their own genotype. Twelve other animals of each type were given cells from rats of the other genotype.

One milliliter of blood was removed from each rat by cardiac puncture 20 μCi $Na_2{}^{51}CrO_4$ (ICN, 651 Ci/g) was added to each sample. After incubation for 70 minutes at 37 °C, the cells were washed three times with 4 vol of sterile saline and suspended in 0.6 mL of warm saline. Labeled cells were reinjected by cardiac puncture into recipients anesthetized with ether. Subsequent samples for radioactive counting were obtained by clipping the tail and collecting the blood in heparinized calibrated pipettes. ^{51}Cr counts were measured in a gamma counter (Model 5500, Beckman Instruments, Irvine, Calif). Serial counts were obtained over a 19-day period, using the value after 24 hours as 100%. Counts were corrected for ^{51}Cr decay[9] and for chromium elution from rat red cells of 1.88% per day.[10] A regression analysis of the log cpm/0.1 mL blood at various times was calculated for each animal and red cell half-life calculated from these regressions. Seven of the 42 animals had a great deal of noise in chromium counts until day 4, after which decay was linear. For these animals, day 0 counts were estimated from the linear points and percentage of survival was recalculated.

RESULTS

Marrow transplantation. Expression of the $^{III}\beta$-globin locus was used as a marker for the donor and recipient cells in cross-transplanted animals. First, the electrophoretic mobility for *AA*, *AB*, and *BB* hemoglobin was determined, using the method of Garrick et al[11] (Fig 1). Variant hemoglobins contain $^{III}\beta$-chains and constitute about 20% of the total hemoglobin.[11] To quantify $^{IIIa}\beta$- and $^{IIIb}\beta$-chain expression, $^{I}\alpha_2{}^{IIIb}\beta_2$ and $^{I}\alpha_2{}^{IIIa}\beta_2$ bands were cut from Ponceau S-stained cellulose acetate strips, dissolved in 3 mL acetic acid:acetone (1:1), and quantified by reading their absorbance at 520 nm. In order to determine the proportions of A and B hemoglobins in specimens from animals with transplants, a standard curve was produced by plotting the values from electrophoreses of mixtures of A and B hemoglobins (Fig 2). Then the proportion of B hemoglobin in the circulating red cells of transplanted animals could be measured and used as a marker for the degree of repopulation of the peripheral blood with cells from the donors.

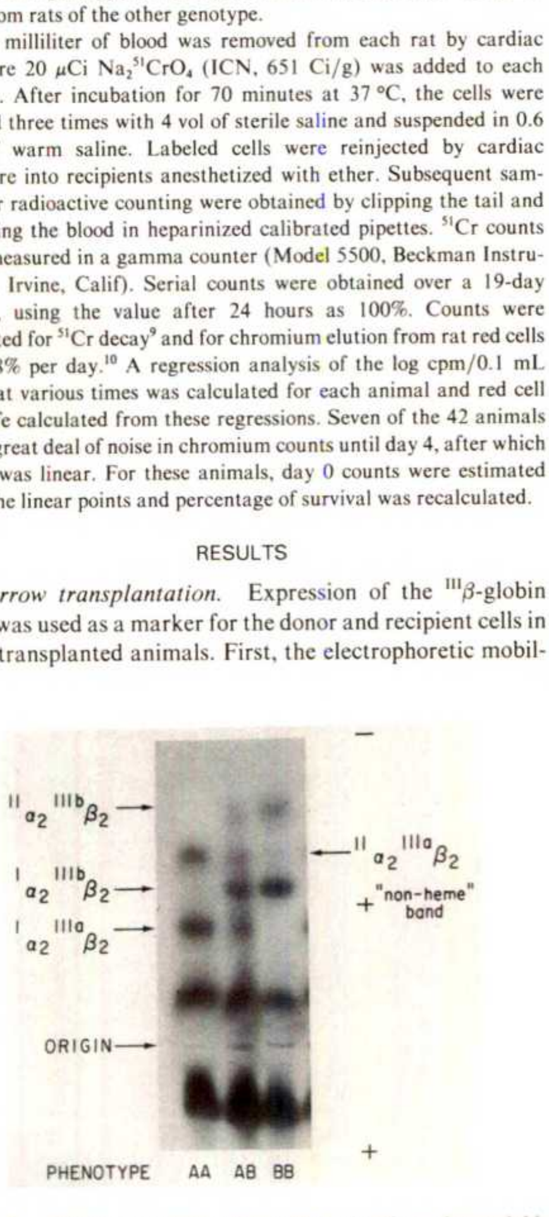
$^{II}\alpha_2{}^{IIIb}\beta_2$
$^{I}\alpha_2{}^{IIIb}\beta_2$
$^{I}\alpha_2{}^{IIIa}\beta_2$
ORIGIN
PHENOTYPE
AA
AB
BB
−
$^{II}\alpha_2{}^{IIIa}\beta_2$
+ "non-heme" band
+

Fig 1. **Cellulose acetate electrophoresis of rat hemoglobin phenotypes AA, AB, and BB with variant hemoglobins indicated.**

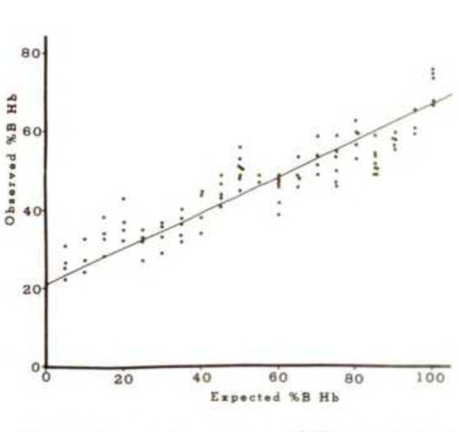

Fig 2. **Plot of observed v expected %B hemoglobin obtained from quantification of A and B hemoglobins after electrophoresis of mixtures of blood of types AA and BB in known quantities. The line is the "best fit" line from a least square regression analysis.**

In Table 1, the genotypes and survival of transplanted animals are shown. Control groups consisted of H animals who were first irradiated and then received H marrow from a single sibling donor (recipient animals are designated H → H); a corresponding L → L control group was also established. The survival of both control groups was 100%. The experimental groups consisted of L animals who received H marrow (H → L) and H animals who received L marrow (L → H). Of 18 animals in the H → L groups, 56% survived; of 14 L → H animals, 64% survived. After transplantation, all H → H and L → L animals displayed only the hemoglobin type of the donor, as expected. After cross-transplantation, the proportion of the circulating cells derived from the donor varied from 0% to 87% for the L → H groups and from 10% to 99% for the H → L groups, as shown

Table 1. Genotypes and Numbers of Animals in Bone Marrow Transplant Groups

Genotype	No. Irradiated	Marrow Genotype	Transplant Type	No. Surviving to 40 Days	Percentage Surviving
DDBB(H)	14	*ddAA*(L)	L→H	9	64
DDBB(H)	15	*DDBB*(H)	H→H	15	100
ddAA(L)	18	DDBB(H)	H→L	10	56
ddAA(L)	15	*ddAA*(L)	L→L	15	100

Table 2. Hematologic Data 40 to 52 Days Posttransplant

	Transplant Type			
	L→H	H→H	H→L	L→L
N	9	15	10	15
Donor cells in circulation (%)	45 (0–87)	—	63 (10–99)	—
Hemoglobin (g/dL)	14.5 ± 26.0	15.1 ± 1.3	15.0 ± 0.9	15.2 ± 1.1
RBC (10^{12}/L)	6.5 ± 0.7	6.8 ± 1.3	7.0 ± 0.4	6.8 ± 0.7
MCV (fL)	67.3 ± 6.5	64.8 ± 4.4	64.6 ± 4.5	57.2 ± 4.9
Reticulocytes (%)	3.4 ± 0.8	3.0 ± 1.7	3.0 ± 1.5	3.2 ± 0.5

in Table 2. However, within 40 to 52 days of transplantation, the hemoglobin, red cell concentrations, MCV, and reticulocyte count had returned to pretransplant values. In cross-transplanted animals, the finding of residual A hemoglobin in the H → L animals or B hemoglobin in the L → H animals indicated that irradiation did not completely eradicate the recipient's marrow. In an attempt to achieve eradication of the recipient marrow, higher doses of irradiation (9.4, 9.7, or 9.8 Gy) were given, but all animals died within four days, exhibiting gut necrosis at postmortem examination.

In order to determine the effect of cross-transplantation on posttransplant *Dpg* genotype, the enzymatic activities of PK and PFK and the red cell concentrations of ATP and DPG were measured. Then, to determine the phenotypic contribution of marrow from either the donor or the recipient, the results were plotted according to the proportion of circulating hemoglobin derived from H (B hemoglobin-producing) sources. The means and ranges (Fig 3) for the H → H rats and L → L rats are included at the extremes of each graph, with data for cross-transplanted animals plotted as a single point for each animal. Regression lines were calculated using only the data for cross-transplanted animals; H → H and L → L rats were not included for this calculation. Data for PK activity correlated closely with the degree of transplant, regardless of the donor or the recipient phenotype ($P < .0001$), as did values for DPG ($P < .001$) and ATP ($P < .01$). PFK activity did not correlate as closely with donor cell phenotype ($P < .05$), but as can be seen (Fig 3), there was considerable variation within each control group and substantial overlap of values for the two (H → H and L → L) control groups.

Red cell survival. In Table 3, the data are shown for ^{51}Cr-labeled red cell survival in four groups of rats. Of the 48 animals transfused, one died during the procedure and five had low counts 24 hours after transfusion. For the remaining 42 animals, the average cpm/0.1 mL blood 24 hours after injection of labeled cells was 10,000 ± 2,465. The mean and standard deviation of regression coefficients (r^2) for animals in each group are high, indicating the expected log-linear decay of label (Table 3). The half-life of L cells in L recipients was 18.7 days; that of H cells in H recipients was 28.6 days. Student's t tests indicated survival of these cells was similar when they were crossed-transfused into recip-

Table 3. ^{51}Cr-Labeled Red Cell Survival

Transfusion Type (donor cell type → recipient genotype)	N	r^2	$T_{1/2}$	
		$\bar{X}$ ± SD	$\bar{X}$ ± SD	Median
L→L	9	0.97 ± 0.05	18.74 ± 3.40	17.2
L→H	10	0.96 ± 0.05	16.10 ± 4.51	16.9
H→H	11	0.94 ± 0.05	26.83 ± 9.78	26.5
H→L	12	0.97 ± 0.02	24.77 ± 5.99	26.2

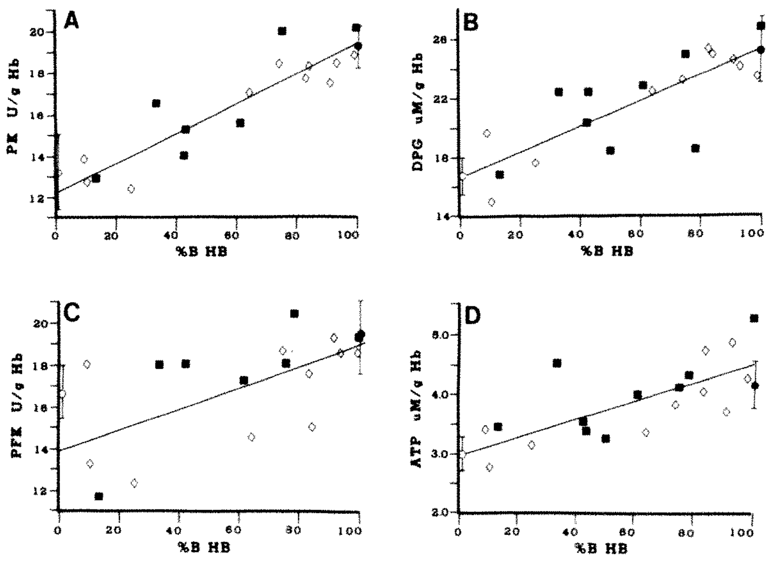

Fig 3. Values for phenotypic markers of *Dpg* genotype: (A) PK and (B) PFK activities and (C) DPG and (D) ATP levels. Open and closed circles are the means with the standard deviation for the L → L and H → H control rat groups, respectively. Diamonds and squares are individual values for the H → L and L → H cross-transplantation groups, respectively. Lines drawn are from the regression analyses using values for H → L and L → H animals only.

ients of differing phenotype. Survival was significantly lower for L cells than for H cells, regardless of recipient phenotype ($P < .05$).

DISCUSSION

Different alleles at the *Dpg* locus are responsible for alterations in the PFK and PK activities of red cells and in concentrations of the glycolytic intermediates, DPG or ATP. That the changes in glycolytic intermediates are secondary to in vivo differences in PFK activity seems clear, since in vivo glycolytic intermediate levels reflect a metabolic block at the PFK step of glycolysis in low-DPG red cells.[4] However, the primary mechanism responsible for the enzymatic alterations is still unknown.

In a broad sense, the *Dpg* gene might act in one of two ways. First, it may be postulated that an extracorpuscular, perhaps a soluble factor in the plasma, causes the glycolytic alterations by its effect on the red cell. For example, studies of uremic patients have shown that plasma phosphate concentrations modify red cell metabolism.[12] Magnesium deficiency in rats results in shortened red cell survival and decreased DPG and ATP levels.[13] Glucocorticoids,[14] catecholamines,[15] and aldosterone[16] are among hormones that modulate DPG metabolism. Another broad category is the consideration of an intracorpuscular product of the *Dpg* gene that might modify glycolysis.

Recent work from this laboratory suggests that the allelic products of the *Dpg* locus act during reticulocyte maturation; reticulocytes from *DD* and *dd* rats do not differ with respect to markers of *Dpg* genotype. As these reticulocytes mature, however, differences characteristic of genotype become apparent.[5] Because the quantity of immunologically detectable PFK does not differ in mature *DD* and *dd* cells,[17] synthesis and degradation of PFK is probably not affected by genotype at this locus. Rather, the PFK present may be altered, perhaps by a secondary modification during reticulocyte maturation, to yield an enzyme with lower specific activity in mature red cells.

The same mechanism by which PFK loses activity in mature red cells may alter the specific activity of PK. Thus, the *Dpg* gene product could be a factor produced only in reticulocytes that acts on both enzymes. Simultaneous alterations in the values for the four markers of genotype at *Dpg* are consistent with the hypotheses that *Dpg* is either a single genetic locus or a cluster of closely linked genes affecting red cell metabolism and survival. Structural loci for PK and PFK are not linked in humans, but until they are mapped in the rat, we cannot rule out the possibility that they are part of *Dpg*.

In the present study, we attempted to distinguish between an extracellular and an intracellular mechanism for *Dpg* by using marrow transplantation and cross-transfusion to observe the effect of host (extracorpuscular) environment on expression of *DPG* phenotype by circulating cells. First, animals received irradiation, followed by transplantation of either sibling (like phenotype) marrow or marrow from donors of differing phenotype. If *Dpg* phenotype expression depends on extracorpuscular factors, it would be expected that recipient rats would have "converted" donor cells to the recipient phenotype. Instead, donor cells, recognized by the closely linked A and B hemoglobin markers, retained their *Dpg* phenotype in a fashion similar to that observed in simple mixing experiments of L and H cells in vitro. This supports the concept that the product of the *Dpg* gene is intracorpuscular. Further conclusions can be taken from the observation that animals who received cross-transplants were invariably chimeric, with circulating red cells from both donors and recipients. Because *Dpg* phenotype correlated directly with the proportions of the two populations, it seems unlikely that the *Dpg* gene produces a factor originating in the marrow that is transferred from a non-erythrocytic cell population to either circulating or developing erythrocytes. Interestingly, the animals have been kept outbred. Commercially obtained Long-Evans rats are regularly typed and introduced into the lines. The fact that so many transplants were successful suggests that, though systematically outbred, Long-Evans rats are immunologically similar, although the period of observation after transplantation was relatively short.

In order to test further the hypothesis that the *Dpg* gene acts intracorpuscularly, we performed cross-transfusion experiments in which survival of H or L red cells was measured in various hosts. The survival of H cells was unaffected by transfusion into L recipients, as was the reverse: transfusion of L cells into H recipients. Because chromium binds to β-globin and these rats differed at the $^{\text{III}}\beta$ locus, it is conceivable that part of the survival difference observed is due to different elution rates from $^{\text{IIIa}}\beta$ and $^{\text{IIIb}}\beta$. Several things suggest that this is not the case. First, these $^{\text{III}}\beta$-containing hemoglobins constitute only about 20% of the total hemoglobin present (Fig 1).[11] Second, in a previous study, autologous transfusion of ^{51}Cr-labeled L or H cells was done using animals of all four possible genotypes (ie, *ddAA*, *ddBB*, *DDAA*, and *DDBB*). Although L cells had shortened survival compared with H cells, there were no differences within groups that could be attributed to different hemoglobin types.[6]

From the present studies, we conclude that the action of the *Dpg* gene is best explained by an intracorpuscular factor.

ACKNOWLEDGMENT

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REFERENCES

1. Noble NA, Brewer J: Identification of a major locus contributing to erythrocyte 2,3-diphosphoglycerate variability in hooded (Long-Evans) rats. Genetics 85:669, 1977

2. Brewer GJ, Gilman J, Noble N, Crews V: Association in Long-Evans hooded rats of red cell 2,3-diphosphoglycerate levels with hemoglobin types. Biochem Genet 16:695, 1978

3. Gilman JG: Red cell phosphofructokinase and pyruvate kinase activities correlate with genetic variation of 2,3-bisphosphoglycerate in rats. Biochem Biophys Res Commun 102:766, 1981

4. Noble NA, Kuwashima LH, Togioka TT, Tanaka KR: Erythrocyte isozymes of phosphofructokinase in genetically high and low 2,3-diphosphoglycerate rats. Biochem Genet 20:1055, 1982

5. Noble NA, Kuwashima LH, Mairbaurl H: Aging of rat erythrocytes: Differences in genetically high and low 2,3-DPG rat lines, in Brewer GJ (ed): Progress in Clinical and Biological Research, vol 165. New York, Alan R. Liss, 1984, p 241

6. Noble NA, Brewer GJ: Studies of the metabolic basis of the ATP-DPG differences in genetically selected high and low ATP-DPG rat strains, in Brewer GJ (ed): Hemoglobin and Red Cell Structure and Function. New York, Plenum, 1972, p 155

7. Noble N: The *Dpg* gene in rats: Studies of mechanism and developmental stage of its action, in Eaton JW, Konzen DK, White JG (eds): Cellular and Molecular Aspects of Aging: The Red Cell as a Model. New York, Alan R. Liss, 1985, p 389

8. Beutler E: Red Cell Metabolism: A manual of biochemical methods (ed 2) Orlando, Fla, Grune & Stratton, 1975, p 10,19

9. Reinhart GD, Lardy HA: Rat liver phosphofructokinase: Kinetic activity under near-physiological conditions. Biochemistry 19:1477, 1980

10. Diem K, Lentner C (eds): Scientific tables, Ciba-Geigy Ltd, Basel, Switzerland, 1970, p 296

11. Jones NCH, Cheney B: The use of ^{51}Cr and ^{59}Fe as red cell labels to determine the fate of normal erythrocytes in the rat. Clin Sci 10:323, 1961

12. Garrick LM, Sharma VS, McDonald MJ, Ranney HM: Rat haemoglobin heterogeneity: Two structurally distinct α chains and functional behavior of selected components. Biochem J 149:245, 1975

13. Summerfield GP, Bellingham AJ, Manlove L, Forbes AM, Goldsmith HJ: Erythrocyte metabolism in patients on haemodialysis and continuous ambulatory peritoneal dialysis. Clin Sci 62:479, 1982

14. Oken MM, Lichtman MA, Miller DR, Leblond P: Spherocytic hemolytic disease during magnesium deprivation in the rat. Blood 38:468, 1971

15. Oimomi M, Yoshimura Y, Kubota S, Tanke G, Takagi K, Baba S: Effect of hydrocortisone on the synthesis of 2,3-diphosphoglycerate in human erythrocytes. Transfusion 22:266, 1982

16. Mairbaurl H, Humpeler E: The influence of adrenaline on the metabolism of erythrocytes in vitro. Biochem Soc Trans 9:99, 1981

17. Boning D, Meier U, Skipka W, Kulpmann WR, Meurer KA: Some evidence for aldosterone action on 2,3-diphosphoglycerate level in human red cells. Metabolism 25:9, 1976

Pro-Urokinase: A Study of Its Stability in Plasma and of a Mechanism for Its Selective Fibrinolytic Effect

By Ralph Pannell and Victor Gurewich

Highly purified pro-urokinase (pro-UK) or single-chain urokinase-type plasminogen activator (scu-PA) was treated with diisopropylfluorophosphate (1mmol/L) to eliminate traces of two-chain UK activity. This preparation was found to retain a low activity against a chromogenic substrate (S2444), equivalent to 0.1% to 0.5% of the activity of its plasmin-activated derivative. Evidence is presented that the intrinsic activity of pro-UK (scu-PA) was sufficient to activate plasminogen on a fibrin plate or in buffer and was far more reactive against Lys-plasminogen than against Glu-plasminogen. The relative resistance of Glu-plasminogen to activation was overcome by the addition of lysine (25 mmol/L) to the reaction mixture. By contrast, in plasma, pro-UK (scu-PA) was stable and nonreactive for >72 hours when incubated (37 °C). Pro-UK (scu-PA) did not form sodium dodecyl sulfate-stable inhibitor complexes, whereas complexation occurred rapidly with UK. Only at high concentrations of pro-UK (scu-PA) (≥250 IU/mL) did plasminogen activation in plasma occur. The relative inertness of pro-UK (scu-PA) in plasma, in contrast to its low-grade enzymatic activity in buffer, was attributed to the effect of inhibitors. The addition of EDTA or the removal of divalent cations by dialysis was associated with a lower threshold for nonspecific plasminogen activation by pro-UK (scu-PA) in plasma. Replacement of Ca^{++} but not other cations restored baseline conditions. In the presence of a clot, fibrin-selective plasminogen activation and clot lysis were triggered. Lysis was accompanied by <10% conversion of pro-UK (scu-PA) to two-chain UK, suggesting that the intrinsic activity of pro-UK (scu-PA) itself may have been responsible for fibrinolysis, although a contribution by the small amount of UK generated could not be excluded. Similarly, pro-UK (scu-PA) supported clot lysis for several days in the same plasma before the effect dissipated as a result of degradation to UK. When Glu-plasminogen in plasma was replaced by Lys-plasminogen, or when lysine was added to normal plasma, nonselective plasminogen activation and fibrinogenolysis occurred. It was concluded that under the experimental conditions, the fibrin specificity of pro-UK (scu-PA) can be explained by its selective activation of fibrin-bound plasminogen and is due to the latter's Lys-plasminogen-like conformation. The free plasminogen in plasma is spared due to its Glu-form as well as to the stability of pro-UK (scu-PA) in the presence of plasma inhibitors and Ca^{++}. This mechanism appears not to require fibrin binding, and clot lysis is accompanied by little activation of pro-UK (scu-PA) to two-chain UK.

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A SINGLE-CHAIN FORM of ~55,000-dalton urokinase (UK) was first purified and identified by Husain et al in our laboratory. It was isolated from urine by affinity chromatography on fibrin/Celite that did not bind other forms of UK.[1-3] Subsequent studies of a single-chain UK plasminogen activator (scu-PA) purified from the fluid of a transformed human kidney cell culture indicated that it was a latent form activatable by plasmin correlating with conversion to two-chain HMW-UK. Nonetheless, the single-chain UK showed a low inherent amidolytic activity.[4] Wun et al[5] and Nielsen et al[6] purified scu-PA from human carcinoma cells in culture, and from its characteristics, concluded that scu-PA was a proenzyme. A latent, trypsin-activatable UK activity was originally detected in kidney cell culture medium by Bernik[7] and by Nolan et al.[8]

The fibrinolytic properties of pro-UK (scu-PA) were compared with UK in vitro and in laboratory animals. A superior fibrin specificity and lytic efficacy were demonstrated, but the mode of action of pro-UK (scu-PA) was not established.[4] Unlike tissue plasminogen activator (t-PA), fibrin clot binding of pro-UK (scu-PA) in plasma was not found, in contrast to fibrin/Celite binding in urine,[1-3] indicating that its fibrin-selective proteolytic effect could not be explained on this basis.[9] The present study was undertaken to investigate alternative mechanisms to fibrin binding to explain the fibrinolytic properties of pro-UK (scu-PA).

The findings indicated that under the experimental conditions, fibrin-selective clot lysis was primarily due to a selective activation of fibrin-bound plasminogen by the intrinsic activity of pro-UK (scu-PA) itself. The effect of inhibitors and Ca^{++} served to prevent the activation of free Glu-plasminogen by pro-UK (scu-PA) in the ambient plasma.

MATERIALS AND METHODS

Pro-urokinase (scu-PA) was purified from the spent tissue culture medium of a transformed human kidney cell line by Collaborative Research, Inc (Lexington, Mass) as previously described.[4] Two-chain UK (55,000-dalton) was a gift from Serono Laboratories (Randolph, Mass). For some experiments, UK was prepared by plasmin conversion of pro-UK (scu-PA) (see later). Antisera to urinary and to tissue culture UK were obtained from Collaborative Research.

Human fibrinogen and amidolytic substrates (S2251 and S2444) were obtained from Kabi (Stockholm); a crude bovine thrombin "thrombostat" preparation was obtained from Parke-Davis (Morris Plans, NJ) and highly purified human thrombin, from Collaborative Research. Streptokinase was a product of Hoechst-Roussell (Somerville, NJ).

Aprotinin (Trasylol) was obtained from FBA Pharmaceuticals (New York). Thromboplastin, diisopropylfluorophosphate (DFP), Tris [Tris(hydroxymethyl) aminomethane] and HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) were obtained from Sigma Chemical Co (St Louis). Bovine serum albumin (BSA), "Purified," was obtained from Calbiochem (La Jolla, Calif).

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Fresh frozen citrated plasma was obtained from St Elizabeth's Hospital Blood Bank. Three units of this stock plasma were pooled and stored in aliquots at −20 °C. Fresh plasma was prepared from the blood of volunteers collected into citrate, and serum from the same donors was prepared from fresh blood collected in glass and incubated (37 °C) for two hours. Plasma or serum was dialyzed (72 hours) against 25 mmol/L HEPES-buffered saline at pH 7.4 to remove divalent cations and chelators.

The integrity of the native form of plasminogen in the stock plasma was ascertained after isolation of the plasminogen (see later) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5% gels alongside Glu- and Lys-plasminogen.

Glu-plasminogen was purified on Lysine-Sepharose essentially by the method of Castellino and Powell[10] from DFP-treated fresh plasma. Lys-plasminogen was prepared from purified Glu-plasminogen by the method of Lucas et al.[11]

Plasmin was prepared by activation of plasminogen (2.5 μmol/L) at pH 8.8 with an equal volume of a 10% suspension of immobilized UK (Affi-Gel 15; Bio-Rad, Richmond, Calif) for 30 minutes at 37 °C.

Plasminogen activator activity was measured on a standard fibrin plate made with human fibrinogen[12] against the World Health Organization International Reference Preparation (IRP) of UK, 66/46 (National Institute of Biological Standards and Control, London). Assignment of unitage (in International Units [IU]) for pro-UK (scu-PA) solutions was based on latent activity, after activation with plasmin and dilution to eliminate significant plasmin (see later).

UK amidolytic activity was measured with Kabi substrate S2444 at 37 °C. The reaction buffer was 0.1 mol/L Tris-HCl (pH 8.8, 0.1 mol/L NaCl) 0.1 mg/mL BSA, with or without aprotinin (200 KIU/mL); and the substrate was 0.75 mmol/L. One enzyme unit (EU) was defined as the amount of enzyme producing a change in absorbance (405 nm) of 1 absorbance unit per minute in 0.4 mL of reaction mixture stopped with 0.4 mL of saline/5% acetic acid. Aprotinin was added (100 KIU/mL) to inhibit plasmin in some samples. When assayed in this system, the IRP-UK had an activity of about 275 EU/100,000 IU.

Plasmin was defined as activity against Kabi substrate S-2251 and assayed in a reaction buffer of 0.1 mol/L Tris-HCl (pH 7.4), 0.1 mol/L NaCl, and 0.1 mg/mL BSA. Plasminogen was activated for assay by preincubation with streptokinase (5,000 U/mL) for 40 minutes at 37 °C.

To measure latent activity, pro-UK (scu-PA) (12,500 IU/mL) was incubated with plasmin (0.1 μmol/L) for 30 to 45 minutes at 37 °C and then diluted for assay on fibrin plate or assayed for amidolytic activity with the addition of aprotinin (200 K IU/mL) to the reaction buffer. Pro-UK (scu-PA) in plasma was measured with S2444 after secondary activation using streptokinase. The concentration was calculated by substracting the UK activity present without activation.

SDS-PAGE was performed by the method of Laemmli,[13] using a 12.5% polyacrylamide gel. Samples were prepared either with or without 10 mmol/L dithiothreitol (DTT). Gels were either stained with silver[14] or dried for autoradiography against Kodak X-Omat R film.

Pro-UK (scu-PA) was labeled with ^{125}I by way of the lactoperoxidase reaction, using immobilized lactoperoxidase-glucose oxidase (Enzymobeads, Bio-Rad). Fifty micrograms of pro-UK in 70 μL of 0.2 mol/L sodium phosphate (pH 7.2) was reacted with 50 μL Enzymobeads suspension, 10 μL (2 mCi) Na ^{125}I (New England Nuclear, Boston), and 25 μL of 1% D-glucose for 15 minutes at room temperature. Free iodine was separated on a column of Sephadex G-10 (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mmol/L sodium acetate (pH 4.8), 0.1 mol/L NaCl, 0.1% Triton X-100 (Sigma, St Louis). The resulting specific activity of the pro-UK (scu-PA) was approximately 30μCi/μg. Labeling was accompanied by some conversion to UK and some loss of enzymatic activity, but the product could be fully cleaved by plasmin at a rate comparable to unlabeled pro-UK (scu-PA). Radiolabeled UK was made by plasmin conversion of ^{125}I-pro-UK (scu-PA).

Immunoprecipitations were performed by the method of Kessler,[15] using *Staphylococcus aureus* cells obtained from Calbiochem (Pansorbin). Samples containing an estimated 200 to 500 ng UK were precipitated with 10 μL of antiserum against urinary UK. The immunoabsorption buffer was 0.1 mol/L Tris (pH 7.2), 0.1 mol/L NaCl, 0.1% Triton X-100. After electrophoresis and autoradiography, the distribution of counts in bands was determined by counting cut sections of the dried gel. The percentage of conversion was calculated and corrected for the two-chain UK contaminant in the probe. Some degradation of the single-chain UK invariably occurred during radiolabeling.

Enzyme treatment of pro-UK (scu-PA). Pro-UK (scu-PA) was activated and converted to its two-chain derivative by treatment with plasmin. Twenty-five microliters of pro-UK (scu-PA) (0.9 μmol/L in 0.1 mol/L Tris, pH 7.4, 0.3 mol/L NaCl) was mixed with 25 μL of plasmin (0.25 μmol/L in the same buffer) and reacted for 45 minutes at 37 °C.

Conversion to UK was evaluated by amidolytic assay and by reducing SDS-PAGE at time points as well as by fibrin plate assay (after >1,000-fold dilution that eliminates any plasmin effect on the assay as determined by control).

Fibrinogen was determined as thrombin-clottable protein.

Analysis of DFP-treated pro-UK (scu-PA). Pro-UK (scu-PA), UK, and plasminogen were treated with 0.01 to 1.0 mmol/L DFP in 0.1 mol/L sodium phosphate (pH 7.2), 0.1 mol/L NaCl, 0.5 mg/mL BSA. Incubation was carried out on ice for 18 hours. For analytical inhibition studies, treated samples were diluted tenfold with 0.1 mol/L Tris (pH 8.8), 0.2 mg/mL BSA, 0.1 mol/L NaCl, and then further incubated and assayed daily until the decay of DFP was complete. For preparative use, the treated product was dialyzed against 10 mmol/L sodium acetate (pH 4.8), 0.15 mol/L NaCl, and stored frozen.

To investigate whether pro-UK (scu-PA) had any enzymatic activity in and of itself, it was first treated with DFP (1 mmol/L) and then assayed with the chromogenic substrate S2444. Assays were done both before and after DFP had substantially decayed in solution. The DFP-treated pro-UK (scu-PA) was also assayed on a standard fibrin plate and in buffer.

Clot lysis induced by pro-UK (scu-PA) or activated pro-UK (scu-PA). ^{125}I-Labeled clots were prepared from stock plasma. Plasma (0.5 mL) plus 1.5 μCi ^{125}I-fibrinogen (Ibrin, human fibrinogen; Amersham Corp, Arlington Heights, Ill) was clotted with thromboplastin and 10 mmol/L $CaCl_2$. The clots were formed in 5-mm (inside diameter) glass tubes, incubated 30 minutes (25 °C), and kept refrigerated overnight as previously described.[4]

These clots were incubated (37 °C) in 3 mL of stock plasma in the presence of added pro-UK (scu-PA) or plasmin-activated pro-UK. At intervals, samples of the incubation medium were removed for measurement of radioactivity and enzymatic activity against chromogenic substrate (S2251). For this, plasma samples were diluted 1:200 and kept on ice. Fibrinogen concentrations were determined after six hours' incubation, on 1-mL aliquots of the lysis supernatant with 2,000 units of aprotinin added.

Effect of Lys-plasminogen v *Glu-plasminogen.* To evaluate the reaction of pro-UK (scu-PA) with Glu- or Lys-plasminogen, each of the reactants was first treated with DFP to eliminate traces of the activated species. The reaction mixture comprised 0.1 μmol/L pro-UK (scu-PA) and 0.1 μmol/L Glu- or Lys-plasminogen in 0.1 mmol/L HEPES (pH 7.4), 0.15 mol/L NaCl, 0.25 mg/mL BSA, 0.75 mmol/L plasmin substrate S2251 ± 25 mmol/L lysine. Reaction mixtures of 100 µL were incubated at room temperature in microtiter plate wells and monitored repeatedly using a Titertek Multiscan microplate reader (Flow Laboratories, Dublin, Va).

Plasminogen-depleted plasma was reconstituted with either Lys- or Glu-plasminogen (0.2 mg/mL). Aliquots (3 mL each) were paired (3 mL each), and standard radiolabeled clots were added to one tube of each pair. Pro-UK (scu-PA) (150 IU/mL) and a radiolabeled probe were added to all four tubes. Clot lysis was followed, and plasminogen was measured at zero time and at completion of clot lysis. At the same time points, samples were obtained for autoradiography of the probe after immunoprecipitation.

Clot lysis with 150 IU/mL pro-UK (scu-PA) was performed in stock plasma to which lysine (0 to 25 mmol/L) was added.

Effect of fibrin on the activation of plasminogen and pro-UK (scu-PA) in plasma. Pro-UK (scu-PA) (200 to 1,000 IU/mL) was incubated in the presence or absence of a standard plasma clot in fresh plasma, serum, or plasminogen-depleted plasma. Clot lysis, UK (S-2444), and plasmin (S-2251) generation were monitored by periodic sampling of the plasma.

Fate of ^{125}I-pro-UK (scu-PA) during clot lysis. ^{125}I-pro-UK (scu-PA) and pro-UK (scu-PA) (100 IU/mL) were added to fresh plasma containing a plasma clot, and the mixture was incubated (37 °C). At intervals, the plasma was sampled until clot lysis was complete. The plasma samples were analyzed by SDS-PAGE under reducing conditions and by autoradiography after immunoprecipitation. Conversion was quantitated by cutting bands from the dried gel and counting them. A ^{125}I-fibrinogen-labeled clot was incubated in parallel and the fractional lysis rate plotted from it.

The conversion of pro-UK (scu-PA) to UK during clot lysis was also studied by measuring the generation of activity against chromogenic substrate S2444 during serial lysis of five and ten clots over a period of five days, incubated in the same plasma. Amidolytic assays for pro-UK (scu-PA) and UK were performed after seven hours' incubation of the plasma mixture. The clots for these experiments were made from fibrinogen (1.5 mg) clotted in the presence of ^{125}I-fibrinogen (1 µCi) with Ca^{++} and thrombin. Two clots were incubated (37 °C) daily in plasma (5 mL) containing pro-UK (scu-PA) (100 IU/mL). Urokinase, pro-UK (scu-PA), and plasminogen were measured in the plasma at baseline and after completion of clot lysis. At the end of each day, the plasma mixture was then refrigerated (4 °C) overnight. The next morning, two new clots were added and the mixture incubated and assays repeated six hours later. This procedure was repeated for five successive days. Bacterial growth was suppressed by antibiotics.

The experiment was repeated with one clot added daily to evaluate the effect of fibrin surface area on the activation of plasminogen and pro-UK (scu-PA).

Effect of divalent cations on clot lysis and nonselective plasmin generation by pro-UK (scu-PA). Pro-UK (scu-PA) (100 IU/mL) or UK (300 IU/mL) were incubated (37 °C) in the following media: stock plasma or serum containing EDTA in a range of concentrations (0.5 to 20.0 mmol/L), serum or stock plasma dialyzed to remove cations and citrate, dialyzed plasma reconstituted with divalent cations, Cu^{++} (20 µmol/L), Zn^{++} (50 µmol/L), Mg^{++} (1 mmol/L), Mn^{++} (1.5 µmol/L) or dialyzed serum reconstituted with Ca^{++} (5 mmol/L) plus or minus the other cations.

Clot lysis, UK, plasmin, and plasminogen were measured. Clots in dialyzed media were made up from dialyzed plasma clotted with thrombin and Ca^{++}.

Degradation of ^{125}I-pro-UK (scu-PA) during clotting. ^{125}I-Pro-UK (scu-PA) (100,000 cpm, ~1 IU) was added to 0.3 mL of freshly drawn human plasma (or buffer) with or without the addition of various inducers of clotting: thromboplastin (Sigma), thrombin (Parke-Davis, Thrombstop), or kaolin. The mixtures were incubated for one hour at 37 °C. Then the clots were centrifuged and the supernatants sampled. Samples of 100 µL were mixed with 25 µL of aprotinin (finally, 1,000 KIU/mL) and 125 µL of 2x immunoabsorption buffer, applied to a 0.2-mL column of protein A-Sepharose (Sigma) to remove endogenous immunoglobulins, and washed through with 1 mL of 1x immunoabsorption buffer to provide the sample for immunoprecipitation and electrophoresis under reducing conditions. The extent of pro-UK (scu-PA) degradation was analyzed by autoradiography.

RESULTS

Enzymatic activation and conversion to UK. Treatment of the pro-UK (scu-PA) with plasmin increased its activity against the amidolytic substrate at a rate dependent on the plasmin concentration. At 0.1 µmol/L plasmin, pro-UK (scu-PA) (0.5 µmol/L) was 93% activated in 15 minutes at 37 °C and was fully activated after one hour. At 0.1 nmol/L plasmin, activation was slow but was extrapolated to completion in about 18 hours (Fig 1). Activation was correlated with conversion to the two chains of UK as determined by reducing SDS-PAGE (Fig 2).

Reaction with DFP. The latent activity of pro-UK (scu-PA) and the expressed activity of UK showed markedly different susceptibilities to inhibition by DFP. When pro-UK (scu-PA) was treated with 1.0 mmol/L DFP, the plasmin-activatable amidolytic activity could be completely recovered after decay of the DFP. On the other hand, UK activity was completely inhibited at 0.02 mmol/L DFP and retained only 5% activity at 0.01 mmol/L DFP.

When pro-UK (scu-PA) was treated with 1 mmol/L DFP, 50 times the concentration needed to inactivate UK, a small amidolytic activity persisted even in the presence of inhibitory levels of DFP. This amidolytic activity was equivalent to about 0.1% to 0.5% of the latent activity after plasmin activation.

When the purified zymogens, pro-UK (scu-PA) and plasminogen, were incubated together in buffer, activation of

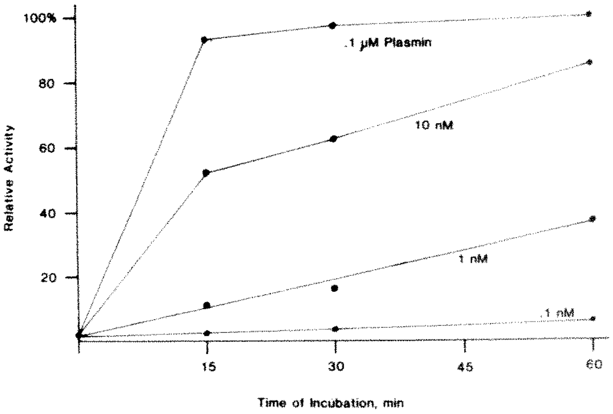

Fig 1. Effect of plasmin treatment on amidolytic activity of pro-UK (scu-PA). Pro-UK (scu-PA) (0.5 µmol/L) was incubated with the indicated concentrations of plasmin in 0.1 mol/L Tris (pH 7.4), 0.1 mol/L NaCl, .001% Tween-80 (Sigma, St Louis). Activity was measured with chromogenic substrate (S-2444) in a reaction buffer containing 200 KIU/mL aprotinin.

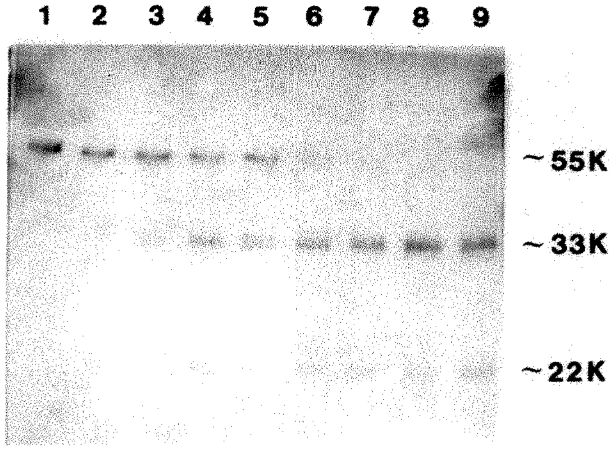

Fig 2. Effect of plasmin (1 nmol/L, lanes 2 through 5; 10 nmol/L, lanes 6 through 9) treatment on molecular form of pro-UK (scu-PA) treated as in Fig 1 and analyzed at 0 (lane 1), 15, 30, 45, and 60 minutes, respectively, by SDS-PAGE under reducing conditions.

plasminogen could be observed after a lag phase that varied with the concentration of the reactants and with the form of plasminogen used (see later).

Fibrin plate assay. The spotting of DFP-treated pro-UK (scu-PA) onto a standard fibrin plate always generated some fibrinolytic activity. On our standard plates made up from human fibrinogen (plasminogen concentration ~0.01 mg/mL final concentration), the specific activity of DFP-treated pro-UK (scu-PA) was about 25,000 IU/mL. Because plasmin is generated in this indirect assay, pro-UK (scu-PA) activation by plasmin cannot be avoided during the course of the assay, resulting in accelerated fibrinolysis due to UK. Untreated pro-UK (scu-PA) preparations (90% to 97% zymogen) had specific activities ranging from 40,000 to 60,000 IU/mg, the higher value being obtained with preparations having a higher percentage of UK contamination. An important additional variable influencing the final result is the plasminogen concentration in the plate, as previously described.[4] Higher specific activities are obtained with higher plasminogen concentrations, presumably due to more rapid activation of pro-UK (scu-PA).

After full activation of pro-UK (scu-PA) with plasmin, described earlier, a specific activity of approximately 100,000 IU/mg was obtained. The latter represents the only way by which the fibrin plate assay of pro-UK (scu-PA) can be made subject to standardizable interpretation.

Clot lysis in plasma by pro-UK (scu-PA) compared with its activated form. A marked difference in clot lysis by pro-UK (scu-PA) and its plasmin-activated derivative was found. At identical concentrations (65 IU/mL), pro-UK (scu-PA) after a lag phase caused accelerated and complete clot lysis, whereas its derivative induced more rapid early lysis that failed to go to completion. Fibrinogen degradation (30%) and detectable plasmin elaboration in the plasma accompanied the latter but was not seen with pro-UK (scu-PA) (Fig 3).

Stability of ^{125}I-pro-UK (scu-PA) in plasma. Autoradiography of ^{125}I-pro-UK (scu-PA) (~1 nmol/L) incubated (37 °C) for four days in plasma or serum showed no change in its appearance from baseline. By contrast, incubation of ^{125}I UK (~1 nmol/L) (activated pro-UK) (scu-PA) resulted in the formation of higher molecular weight bands representing complexes with plasma protease inhibitors. The failure of UK to react completely reflects the incomplete stability of the enzymatic activity to labeling (Fig 4). Stability of the single-chain form of pro-UK (scu-PA) was also shown in buffer (0.1 mol/L HEPES, pH 7.2) over four days at 37 °C.

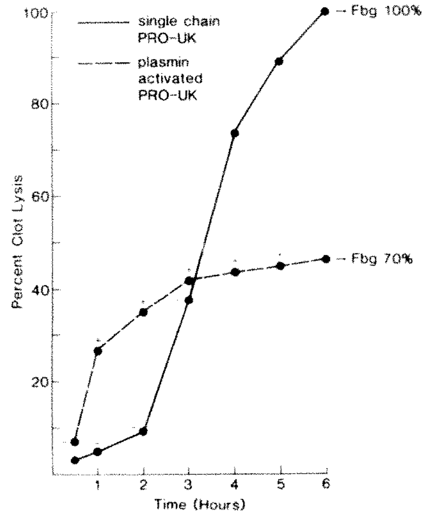

Fig 3. Curves of clot lysis induced by 65 IU/mL of pro-UK (scu-PA) or its activated derivative. Associated enzymatic activity (against S-2251) detectable (+) or undetectable (−) at intervals in the ambient plasma and fibrinogen (percentage of B.L. value) degradation at six hours are shown.

Stability of pro-UK (scu-PA) during clot lysis in plasma. Examination of autoradiographs of reduced SDS-gels of ^{125}I-pro-UK (scu-PA) incubated in plasma during lysis of

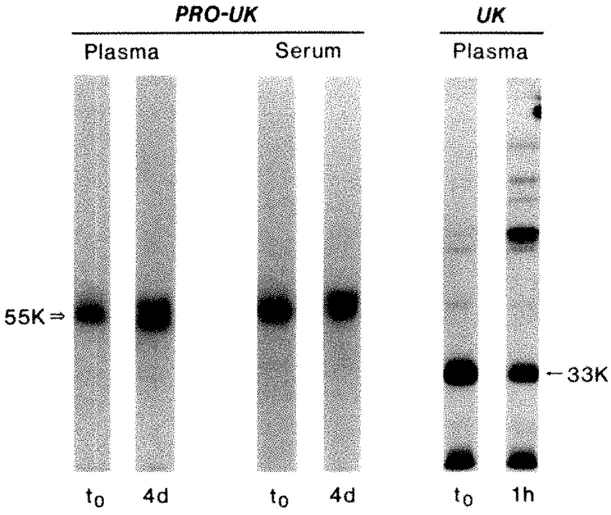

Fig 4. Reduced SDS-PAGE and autoradiography of ^{125}I-labeled pro-UK (scu-PA) or its activated derivative (UK) in plasma or serum after incubation (37 °C). Higher-mol-wt bands representing inhibitor complexes are seen with UK but not with pro-UK (scu-PA), which retained its single-chain structure.

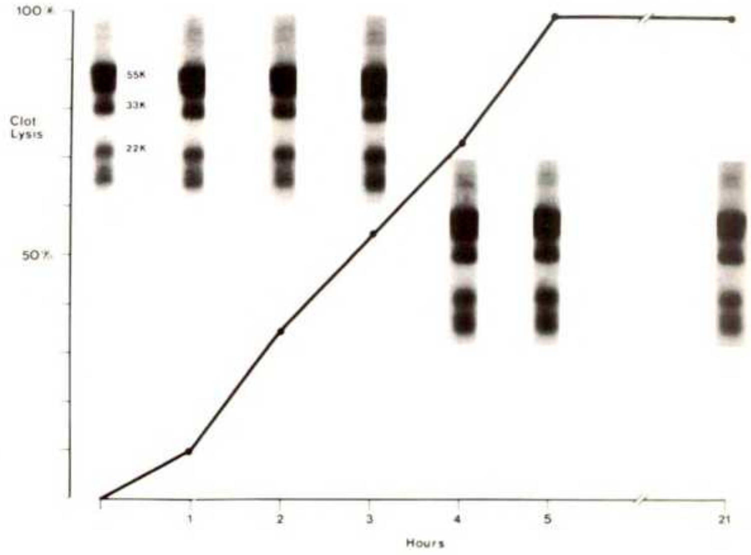

Fig 5. Fate of a ^{125}I pro-UK (scu-PA) probe during lysis of a standard clot by 150 IU/mL pro-UK (scu-PA). The autoradiographic appearance of the probe before and during clot lysis (zero to five hours) and at 21 hours did not change.

a single clot by pro-UK (scu-PA) (150 IU/mL) showed no apparent conversion of the pro-UK (scu-PA) probe. Some contamination by two-chain UK is seen at baseline (B.L.) due to degradation during radiolabeling (Fig 5).

More precise measurements showed only slight conversion. Counting of the sliced gels at t_o gave counts corresponding to 73% and 27% for single- and two-chain UK, respectively. In the control with no clot, after seven hours' incubation, there was no change in this composition of the probe. However, in the presence of a clot, almost 10% conversion accompanied complete clot lysis.

When serial clot lysis with pro-UK (scu-PA) (100 IU/mL) was performed with two clots added daily to the same plasma, there was no significant conversion of pro-UK (scu-PA) to UK by amidolytic assay (S2444 before and after SK activation) until after the second day, at which time 6 mg of fibrin had lysed. Thereafter, a gradual conversion to UK was seen that did not go to completion, even though plasmin elaboration was undoubtedly potentiated by fibrin degradation products. By the end of the fifth day, 20% of the pro-UK (scu-PA) still remained. The total enzyme expression (pro-UK [scu-PA] and UK) was increased after the third day, probably due to some consumption of inhibitors. In the control tubes containing no clots, far less activation of pro-UK (scu-PA) was seen, with >60% remaining at the end of day 5 (Fig 6). When only a single clot was added instead of two clots, the conversion to UK was about half as rapid.

No significant plasmin generation or plasminogen consumption in the plasma during serial clot lysis with two clots was seen until days 4 and 5, when the losses were 40% and 50% of baseline values, respectively. Because fibrin degradation products potentiate plasmin elaboration in this assay, these results may underestimate plasminogen losses. In the control tubes without clots, plasminogen concentrations remained unchanged for five days.

The fractional clot lysis rate for the first three days (3 mg fibrin per day) remained constant. By day 4, clot lysis was retarded and did not go to completion (Fig 7). On the fifth day, no lysis took place.

Effect of fibrin clots on the activation of pro-UK (scu-PA) and plasminogen in plasma. The proteolytic effect of pro-UK (scu-PA) in plasma was invariably clot-related, but fibrin specificity was seen only at concentrations of pro-UK (scu-PA) up to <250 IU/mL. Within this range, clot lysis was unaccompanied by detectable plasmin or UK generation

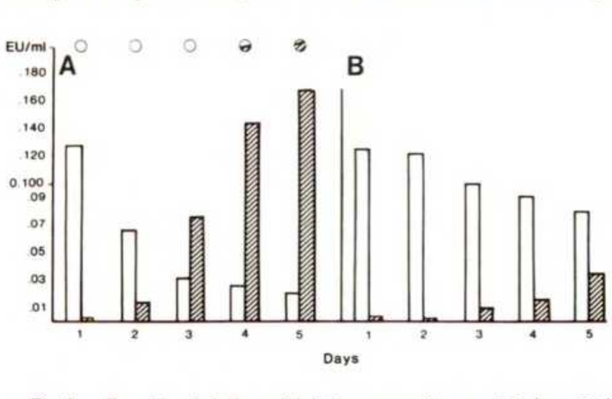

Fig 6. Results of daily amidolytic assays for pro-UK (scu-PA) (open bars) and UK (hatched bars) during serial clot lysis (two clots per day) by pro-UK (scu-PA) (100 IU/mL) (A) and in control plasma without clots (B). The daily results of lysis are represented by the circles (open, complete lysis, or hatched, no lysis).

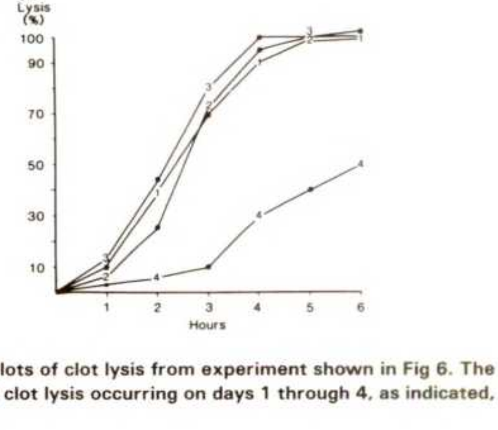

Fig 7. Plots of clot lysis from experiment shown in Fig 6. The dynamics of clot lysis occurring on days 1 through 4, as indicated, are shown.

or by fibrinogen degradation. At higher concentrations (250 to 500 IU/mL), some plasmin generation in the ambient plasma accompanied clot lysis, but this was not detectable in the absence of a clot. At concentrations of 500 to 1,000 IU/mL, plasmin and UK were generated spontaneously and simultaneously after one to three hours' incubation, but their level was considerably potentiated by the addition of a clot. In plasminogen-depleted plasma, no UK generation occurred, indicating that plasmin was responsible for pro-UK (scu-PA) activation under these experimental conditions (Fig 8).

When the experiments were done in serum, the fibrin selectivity of the proteolytic effect of pro-UK (scu-PA) was retained at concentrations that were about 20% to 30% higher than in corresponding citrate plasma.

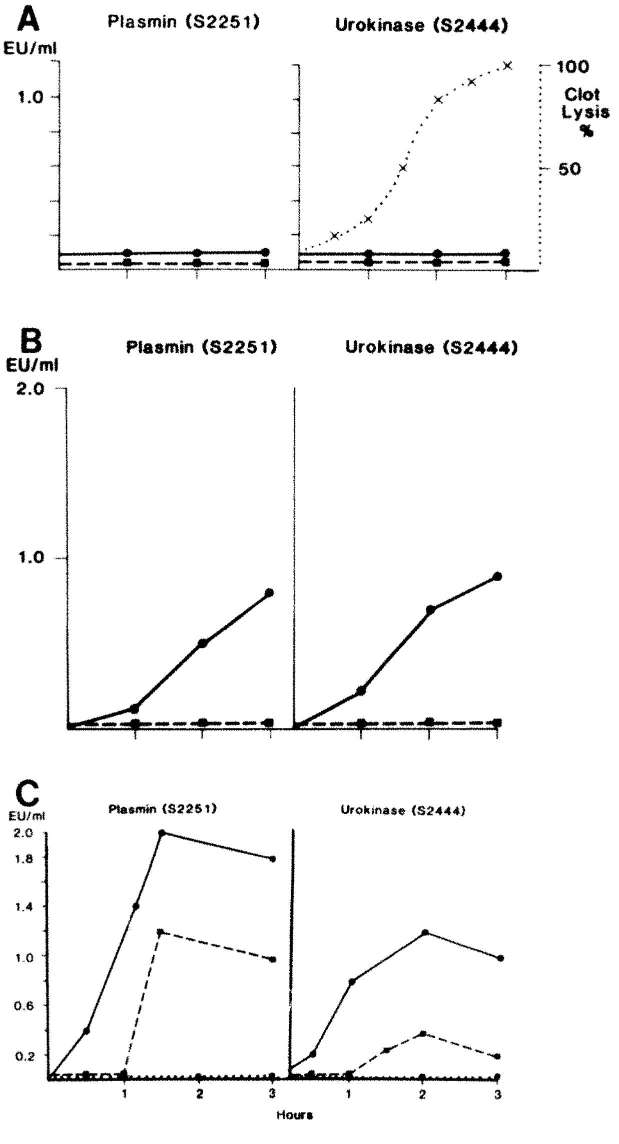

Fig 8. Effect of fibrin clot on plasmin and UK generation by pro-UK (scu-PA) (200 to 1,000 IU/mL in plasma. Enzyme elaboration with clot (●——●) or without clot (■--■) is shown for pro-UK (scu-PA) 200 IU/mL (A), 500 IU/mL (B), and 1,000 IU/mL (C). A plot of clot lysis (X· · · ·X) in the absence of UK or plasmin generation is shown (A). The findings in plasminogen-depleted plasma (●–●) (C) are shown.

Effect of Lys-plasminogen v *Glu-plasminogen.* When purified, DFP-treated pro-UK (scu-PA) and plasminogen were mixed in a buffer system in the presence of plasmin substrate (S2251), there was a mutual interaction resulting in the generation of plasmin after a delay.

Lys-plasminogen was considerably more reactive to pro-UK (scu-PA) than was Glu-plasminogen. The latter reaction had a longer delay phase and did not reach linearity after 50 minutes, whereas the reaction with Lys-plasminogen reached linearity within 15 minutes. The linear phase of the curve indicates that the plasminogen has been fully activated. In the presence of lysine (25 mmol/L), an acceleration of the activation of Glu-plasminogen by pro-UK (scu-PA) occurred, resulting in the two substrates reacting equivalently. A slight delay in the activation of Lys-plasminogen was seen due to the inhibitory effect of lysine (Fig 9).

Clot lysis by pro-UK (scu-PA) (200 IU/mL) in plasma reconstituted with Lys-plasminogen started rapidly and then slowed without going to completion, resembling the clot lysis curve of activated pro-UK (scu-PA), shown in Fig 3. Moreover, a clot lysis in the presence of Lys-plasminogen was associated with a loss of 90% of the plasma plasminogen and conversion of most of the pro-UK (scu-PA) to two-chain UK, as shown in autoradiographs of a radiolabeled pro-UK (scu-PA) probe incubated in the same plasma. In the matched plasma containing Glu-plasminogen, clot lysis followed the dynamics characteristic of pro-UK (scu-PA) and was not associated with plasminogen loss or activation of pro-UK (scu-PA) (Fig 10).

In control tubes without clots, pro-UK (scu-PA) in Lys-plasminogen–reconstituted plasma induced comparable plasminogen consumption and two-chain UK generation as with a clot. Therefore, in the presence of Lys-plasminogen, the fibrin clot selectivity of the proteolytic effect of pro-UK (scu-PA) in plasma was lost. A comparable nonspecific

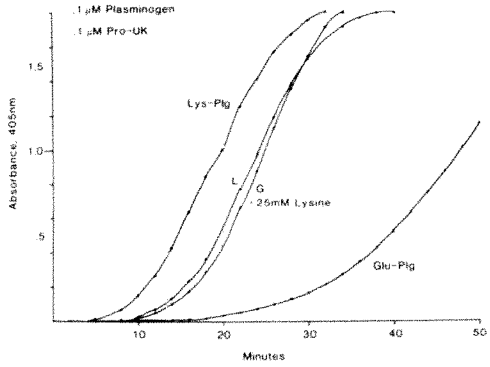

Fig 9. The reaction of DFP-treated pro-UK (scu-PA) and plasminogen in buffer as monitored by the accumulation of plasmin chromogenic product (see Materials and Methods). Lys-plasminogen (Lys-Plg) produced an earlier burst of plasmin activity than did Glu-plasminogen (Glu-Plg), which did not reach linearity. In the presence of 25 mmol/L lysine, Glu-plasminogen (G) and Lys-plasminogen (L) behaved approximately the same. The shift to the right of L (relative to Lys-Plg) is due to the inhibition of UK by lysine.

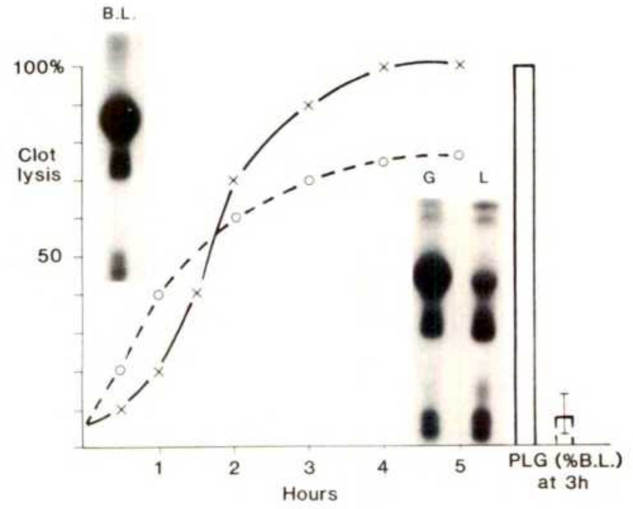

Fig 10. Plots of clot lysis by pro-UK (scu-PA) (200 IU/mL) in the presence of Glu- (×—×) or Lys- (O--O) plasminogen-reconstituted plasma. Autoradiographs of the radiolabeled pro-UK (scu-PA) probe show its composition at baseline (B.L.) and after five hours' incubation in Glu- (G) or Lys- (L) plasminogen/plasma. The single-chain pro-UK (scu-PA) and the heavy and light chains of UK are the major bands shown. The UK contamination at B.L. is due to degradation of pro-UK (scu-PA) during radiolabeling. The concentration of plasminogen (Plg) expressed as a percentage of B.L. at three hours in Glu- (solid line bar) or Lys- (broken-line bar) plasminogen/plasma is shown.

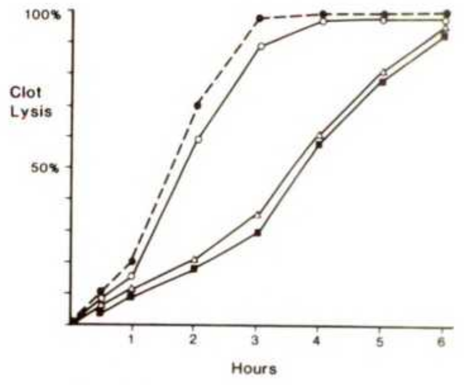

Fig 11. Effect of divalent cations on clot lysis by pro-UK (scu-PA) (300 IU/mL). Clot lysis plots in dialyzed serum (●--●), dialyzed serum + M⁺⁺ (O—O), dialysed serum + M⁺⁺ + 5 mmol/L Ca⁺⁺ (△—△), and in control serum (■—■).

plasminogen activation by pro-UK (scu-PA) occurred when lysine (1, 5, or 25 mmol/L) was added to normal plasma. At 25 mmol/L lysine, a 95% depletion of plasminogen took place in three hours (similar to that in Fig 10).

Effect of divalent cations on pro-UK (scu-PA)-induced clot lysis and on its selective fibrinolytic effect. Increasing concentrations (1 to 10 mmol/L) of EDTA added to serum caused a concentration-dependent potentiation of clot lysis by a small concentration of pro-UK (scu-PA) (15 IU/mL), with only 10% clot lysis in six hours in the absence of EDTA and 100% clot lysis with 10 mmol/L EDTA. A similar potentiation of clot lysis by EDTA was found with UK. However, with pro-UK (scu-PA), the addition of EDTA considerably reduced its fibrin specificity. This potentiating effect on clot lysis at the cost of selectivity could also be induced by EDTA (Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N^1,N^1-tetraacetic acid, Sigma, St Louis) or high concentrations of citrate.

Similar findings were obtained when divalent cations were removed from plasma or serum by dialysis. Reconstituting the dialyzed serum (or plasma) with divalent cations (M^{++}) other than Ca^{++} had little effect. When Ca^{++} (5 mmol/L) was added to dialyzed serum, clot lysis was restored to predialysis kinetics (Fig 11) and nonselective plasminogen activation that occurred in the absence of Ca^{++} was inhibited and selectivity was restored (Fig 12).

Conversion during clotting. Partial conversion of radiolabeled pro-UK (scu-PA) to two-chain UK was seen during clotting, the extent of which varied with the conditions. Without additions, the clotting of native plasma in uncoated glass tubes caused 37% conversion; with thrombin added, 37%; and with thromboplastin, 41%. When clotting of native plasma was stimulated by kaolin, 60% conversion to UK was seen. The addition of kaolin to citrated plasma containing pro-UK (scu-PA) induced 16% conversion, whereas no conversion was seen in the absence of kaolin under these conditions.

DISCUSSION

The persistence of amidolytic activity in pro-UK (scu-PA), after treatment with sufficient DFP to inactivate trace UK contamination, is consistent with our earlier finding that ^{14}C-DFP could be incorporated into the molecule.[3] A reaction with DFP, indicative of an available active site, has also been found in other zymogens, such as trypsinogen, and is not incompatible with its classification as a proenzyme.[16]

The intrinsic activity of pro-UK (scu-PA) was found to be sufficient to activate its principal substrate in buffer or on a fibrin plate. Pro-UK was far more reactive with the Lys-form of plasminogen than with the Glu-form. Such a difference in reactivity is not unprecedented, as it has also been reported for UK.[17,18] The relative resistance of Glu-plasminogen to activation by pro-UK (scu-PA) was overcome by the addi-

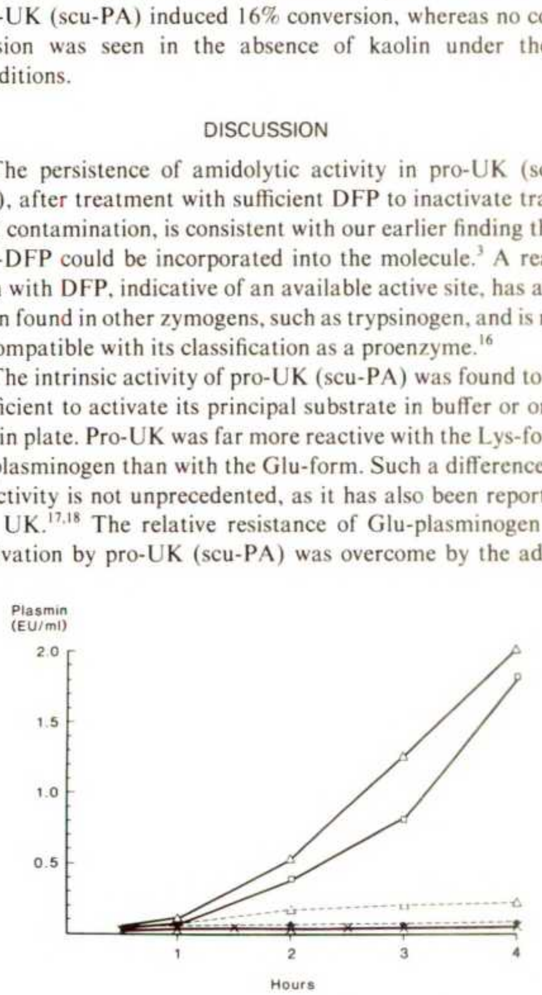

Fig 12. Effect of Ca⁺⁺ on plasmin generation (enzyme units per milliliter) by pro-UK (scu-PA) (300 IU/mL) after incubation of pro-UK (scu-PA) in dialyzed serum (△—△), dialyzed plasma (□—□), dialyzed serum + Ca⁺⁺ (△--△), control serum (●--●), and control plasma (X—X).

tion of lysine to the reaction mixture. This effect of lysine can be explained by its interaction with a lysine binding site on Glu-plasminogen, which induces a conformational change, making Glu-plasminogen functionally similar to Lys-plasminogen.[19,20]

On a fibrin plate, the specific activity of pro-UK (scu-PA) is variable and not subject to standardizable interpretation. For DFP-treated pro-UK (scu-PA), the specific activity was equivalent to about 25% of that of UK, and in the presence of 3% to 10% UK contamination, the specific activity was 40% to 60% of UK. The plasminogen concentration also affects the results obtained by this assay.[4] The final result is largely determined by the extent of pro-UK (scu-PA) activation by plasmin. It is therefore recommended that the fibrin plate assay of pro-UK (scu-PA) be used only as a measure of latent units, ie, after its full activation by plasmin, which gave a specific activity of about 100,000 IU/mg.

In plasma, pro-UK (scu-PA) fibrinolytic capacity was stable for several days, as previously shown,[4] and, unlike UK, did not form SDS-stable inhibitor complexes. At concentrations of ≤ 250 IU/mL, pro-UK (scu-PA) did not activate native plasminogen, in contrast to the activation seen in a purified system, indicating that its intrinsic activity was neutralized by plasma inhibitors. When lysine was added or when native Glu-plasminogen in plasma was replaced by Lys-plasminogen, plasmin generation by pro-UK (scu-PA) occurred.

The depletion of divalent cations by the addition of EDTA or by dialysis reduced the threshold at which activation of native plasminogen by pro-UK (scu-PA) took place in plasma. This effect was shown to be attributable specifically to Ca^{++}, as reconstitution of dialyzed serum by Ca^{++}, and not by other divalent cations, restored baseline conditions.

The addition of a fibrin clot to plasma containing pro-UK (scu-PA) triggered the activation of plasminogen. Plasmin generation was not detectable in the ambient plasma at lower concentrations of pro-UK (scu-PA) (≤ 250 IU/mL). At higher plasma concentrations of pro-UK (scu-PA), the addition of a clot initiated detectable plasmin generation, fibrinogenolysis, and pro-UK (scu-PA) activation. Pro-UK (scu-PA) conversion to UK was not found when plasminogen-depleted plasma was used, indicating that pro-UK (scu-PA) activation was induced by plasmin. At still higher concentrations of pro-UK (scu-PA) (>500 IU/mL), native plasminogen activation and fibrinogenolysis occurred in plasma spontaneously, but these reactions were augmented by the addition of a fibrin clot.

When Glu-plasminogen was replaced by Lys-plasminogen in plasma, the fibrin specificity of clot lysis by pro-UK (scu-PA) was lost. Nonselective plasminogen activation occurred at low concentrations of pro-UK (scu-PA). In contrast to normal plasma, clot lysis was accompanied by a major conversion of pro-UK (scu-PA) to two-chain UK and by fibrinogenolysis. The clot lysis curve in plasma reconstituted with Lys-plasminogen resembled that seen with UK, having a rapid onset and then arresting before lysis was completed. Clot lysis by pro-UK (scu-PA) characteristically starts slowly, accelerates, and goes to completion if sufficient time is given.[4] Nonselective activation of plasminogen by pro-UK (scu-PA), similar to that seen in the presence of Lys-plasminogen, was also seen when lysine was added to normal plasma.

These results in plasma parallel the findings in the purified system of a preferential activation of Lys-plasminogen over Glu-plasminogen or of a "pseudo-Lys" form induced by binding of Glu-plasminogen to a lysine residue. Glu-plasminogen is similarly bound to a lysine residue on fibrin,[21] resulting in a comparable conformational change. The selective activation of fibrin-bound plasminogen by pro-UK (scu-PA) can therefore be explained on this basis. Once plasminogen is activated on the fibrin surface, plasmin will induce modifications in fibrin that expose additional plasminogen binding sites.[22] By this mechanism, more substrate can become available for activation by pro-UK (scu-PA), perhaps explaining the accelerated clot lysis that follows a slow initial phase, characteristic of pro-UK (scu-PA)-induced clot lysis.

Determination of the kinetics of the pro-UK–Glu-plasminogen interaction is complicated by the effect of the reaction product (plasmin) on both "enzyme" (pro-UK) (scu-PA) and substrate (Glu-plasminogen). The result is a generation of UK and Lys-plasminogen that must be scrupulously prevented, since even trace amounts will augment plasmin formation considerably. The problem of maintaining the integrity of the reactants during this reaction has thwarted efforts to determine the true kinetics of pro-UK (scu-PA) itself with undegraded native plasminogen. However, the present findings demonstrate that Glu- and Lys-plasminogen have different reaction susceptibilities to pro-UK (scu-PA).

Fibrin binding of pro-UK (scu-PA) has not been demonstrated in citrate plasma, although it was the property of fibrin affinity that was responsible for the first isolation, purification, and characterization of pro-UK (scu-PA).[1-3] This discrepancy may have an explanation in the recent finding by Husain in our laboratory that the fibrin affinity of pro-UK (scu-PA) is dependent on Zn^{++}.[23] In our model system, Zn^{++} is largely chelated by citrate, whereas urine, the original source of pro-UK (scu-PA),[1-3] is relatively rich in Zn^{++}. In any event, the present findings concern those elements of the mechanism of pro-UK's fibrin selectivity that are independent of direct fibrin binding.

Little conversion of pro-UK (scu-PA) to UK ($<10\%$) during clot lysis was demonstrated. Moreover, the fibrinolytic effect was fully preserved for several days during the serial lysis of six clots, showing a remarkable conservation not only of pro-UK (scu-PA), but also of plasminogen. These findings suggest that under the experimental conditions, lysis may have been maintained by pro-UK (scu-PA) itself, although some contribution by its activated derivative cannot be excluded, since generation of a small amount of UK was seen. Under physiologic conditions, at the low concentration at which pro-UK (scu-PA) is found in blood (5 to 10 $\mu g/L$),[24] pro-UK (scu-PA) activation is more apt to be a precondition for clot lysis. Stimulation of the contact phase of coagulation was shown to activate pro-UK (scu-PA) and may help to trigger a mutual activation of pro-UK (scu-PA) and plasminogen, as previously suggested.[4] The increased susceptibility of fibrin-bound plasminogen to activation by pro-UK (scu-PA) or by UK, as well as any local conditions that favor fibrin binding of pro-UK (scu-PA), should help to promote the fibrin surface as a locus of this reaction.

In conclusion, pro-UK (scu-PA), under the experimental conditions, maintained clot lysis that was accompanied by little (<10%) conversion to UK. The fibrin specificity of pro-UK (scu-PA) in plasma was shown to be related to a selective activation of fibrin-bound plasminogen (pseudo-Lys), a mechanism that is independent of the fibrin-binding property of pro-UK (scu-PA).[9] The resistance of plasminogen in the ambient plasma to activation is attributed to its Glu-form and the stabilizing effect of plasma inhibitors and Ca^{++}.

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REFERENCES

1. Husain SS, Lipinski B, Gurewich V: Isolation of plasminogen activators useful as therapeutic and diagnostic agents (single-chain, high-fibrin affinity urokinase). U.S. Patent No. 4 381 346 (filed Nov 13, 1979; issued Apr 26, 1983)

2. Husain SS, Gurewich V, Lipinski B: Purification of a new, high molecular weight form of urokinase from urine. Thromb Haemost 46:11, 1981 (abstr)

3. Husain SS, Gurewich V, Lipinski B: Purification and partial characterization of a single-chain, high molecular weight form of urokinase from human urine. Arch Biochem Biophys 220:31, 1983

4. Gurewich V, Pannell R, Louie S, Kelley P, Suddith RL, Greenlee R: Effective and fibrin-specific clot lysis by a zymogen precursor form of urokinase (pro-urokinase). A study in vitro and in two animal species. J Clin Invest 73:1731, 1984

5. Wun T, Ossowski L, Reinch E: A proenzyme form of urokinase. J Biol Chem 257:7262, 1982

6. Nielsen LS, Hansen JG, Skriver L, Wilson EL, Kaltoft K, Zeuthen J, Dano K: Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. Biochemistry 21:6410, 1982

7. Bernik MB: Increased plasminogen activator in tissue culture after fibrin deposition. J Clin Invest 52:823, 1973

8. Nolan C, Hall LS, Barlow GH, Tribby, IIE: Plasminogen activator from human embryonic kidney cell cultures. Evidence of a proactivator. Biochim Biophys Acta 496:384, 1977

9. Gurewich V, Pannell R: The fibrin-specificity of single-chain urokinase induced proteolysis is not dependent on fibrin binding. Thromb Haemost 50:1222, 1983 (abstr)

10. Castellino J, Powell JR: Human plasminogen. Methods Enzymol 80:365, 1981

11. Lucas MA, Fretto LJ, McKee PA: The binding of human plasminogen to fibrin and fibrinogen. J Biol Chem 258:4249, 1983

12. Brakman P: Fibrinogen: A standardized fibrin plate method and a fibrinolytic assay of plasminogen. Scheltema, Holkema en Vermeulen, Amsterdam, 1967

13. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature 227:680, 1970

14. Oakley BR, Kirsch DR, Morris NR: A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105:361, 1980

15. Kessler SW: Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. J Immunol 117:1482, 1976

16. Morgan PH, Robinson NC, Walsh KA, Neurath H: Inactivation of bovine trypsinogen and chymotrypsinogen by diisopropylphosphorofluoridate. Proc Nat Acad Sci USA 69:3312, 1972

17. Lucas MA, Straight DL, Fretto LJ, McKee PA: The effects of fibrinogen and its cleavage products on the kinetics of plasminogen activation by urokinase and subsequent plasmin activity. J Biol Chem 258:12171, 1983

18. Lijnen HR, van Hoef B, Collen D: Influence of cyanogen-bromide-digested fibrinogen on the kinetics of plasminogen activation by urokinase. Eur J Biochem 144:541, 1984

19. Violand BN, Sodetz JM, Castellino FJ: The effect of epsilon aminocaproic acid on the gross conformation of plasminogen and plasmin. Arch Biochem Biophys 170:300, 1975

20. Clayes H, Molla A, Verstraete M: Conversion of NH_2-terminal glutamic acid to NH_2-terminal lysine human plasminogen by plasmin. Thromb Res 3:515, 1973

21. Ploplis VA, Cummings HS, Castellino FJ: Monoclonal antibodies to discrete regions of human Glu_1-plasminogen. Biochemistry 21:5891, 1982

22. Harpel PC, Chang T-S, Verderber E: Tissue plasminogen activator and urokinase mediate the binding of glu-plasminogen to plasma fibrin I. Evidence for new binding sites in plasmin-degraded fibrin I. J Biol Chem 260:4432, 1985

23. Husain S: Requirement of Zn^{2+} for a binding interaction between urokinase-type plasminogen activator and fibrin. Tenth International Congress on Thrombosis and Haemostasis. Thromb Haemost 54:724, 1985 (abstr)

24. Wun TC, Schleuning WD, Reich E: Isolation and characterization of urokinase from human plasma. J Biol Chem 257:3276, 1982

Characterization of Murine Peritoneal Macrophage Receptors for Fibrin(ogen) Degradation Products

By Shrin Rajagopalan and Salvatore V. Pizzo

The binding of human fibrinogen degradation fragments D_1, E, X, and Y, as well as fibrin fragment D_1 dimer, to mouse peritoneal macrophages was examined. A Scatchard plot of fragment D_1 binding was biphasic, suggesting two classes of receptors. Fragments D_1, D_1 dimer, X, and Y in low concentrations bound to macrophages with high affinity (K_d = 23 to 73 × 10^{-11} mol/L). Fragment E bound specifically but at a much lower level than the other fragments. Fragment D_1 was able to compete for the binding of radiolabeled fragments X and Y but not radiolabeled fragment E. These studies indicate that fragments D and E are recognized by separate receptor systems but that all of the fibrinogen degradation products that contain the D domain are recognized by the same receptor system.

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PLASMIN DIGESTION of human fibrinogen produces a set of terminal degradation products termed fragments A, B, C, D, and E.[1] Intermediate products X and Y have also been described.[2] Fragment X is produced by digestion of the carboxyl termini of the $A\alpha$-chains of fibrinogen. Fragments A, B, and C, derived from the $A\alpha$-chains, are released during this reaction. Fragment X is further degraded into fragment D and fragment Y, and fragment Y is in turn degraded into fragment E and another fragment D.[3] Because of continued proteolysis, a series of fragment D species may be obtained, termed fragments D_1, D_2, D_3, etc.[3,4] The presence of calcium favors the formation of fragment D_1.[5] Plasmin digestion of cross-linked fibrin, as opposed to fibrinogen, results in fragment D_1 dimer, consisting of two D moieties with cross-linked gamma chains, and fragment E.[3]

The clearance of fibrinogen and fibrinogen fragments from the circulation of animals occurs in the liver and kidneys.[6] A specific clearance pathway for fragment D has been demonstrated in the mouse.[7] Fragments X and Y compete for this pathway, although fragment E does not, suggesting that the recognition site is contained in the D domain, which is shared by fragments X and Y.[8] Autoradiographic studies show a majority of radiolabeled fragment D to localize in the liver, with approximately equal counts seen per hepatocyte or reticuloendothelial cell.[7]

The hepatic synthesis of fibrinogen, an acute-phase reactant, is stimulated by plasmin-derived fragments of fibrinogen and fibrin.[9-11] Franks et al[12] showed that fragment D, but not fragment E, could stimulate fibrinogen synthesis when perfused into rats. It has recently been shown that fragments D and E stimulate fibrinogen synthesis in rat hepatocyte monolayers, not through a direct effect, but through the elicitation of a regulatory protein from blood monocytes.[13] This regulatory protein, hepatocyte-stimulating factor also is released from isolated rat liver macrophages (Kupffer cells) after exposure to fragments D and E.[14]

In the present study, we further characterize the macrophage receptor for fragment D. We also examine the binding behavior of fragment D_1 dimer and investigate for the possible presence of receptors that are capable of binding fragments E, X, and Y. By use of competition studies, it is determined whether these fragments are all recognized by the D receptor previously observed by in vivo methods.

MATERIALS AND METHODS

Fragment D_1 was prepared from human fibrinogen (Kabi Co, Stockholm) as previously described.[3] Fragment E was obtained as a high-salt eluent from the same DEAE-Sephacel ion-exchange column used to obtain fragment D_1. Although ion-exchange chromatography has been the method of choice for purification of fragment E,[2,15] we observed a higher molecular weight (mol wt) contaminant by analytic gel filtration high-performance liquid chromatography (HPLC; Fig 1) on an LKB Ultropac TSK G3000 SW gel filtration column. Removal of this contaminant required gel filtration on a Sephadex G-100 column, followed by HPLC ion-exchange chromatography on an LKB Ultropac TSK 545 DEAE column. Fragments X and Y were prepared as previously described,[2] except that Sephacryl S-300 was used instead of Sephadex G-200 for the gel filtration step. Plasminogen was obtained from human plasma by affinity chromatography on lysine-Sepharose, as described by Deutsch and Mertz[16] and modified by Brockway and Castellino[17] to include gradient elution. Plasminogen was activated with urokinase (Calbiochem, San Diego, Calif).

Protein concentrations in purified preparations of fibrinogen and fibrinogen fragments were determined from the following molar extinction coefficients and mol wt[2]: fibrinogen, $A^{1\%}/1$ cm, 280 nm = 15.1, M_r = 320,000; fragment X, $A^{1\%}/1$ cm, 280 nm = 14.2, M_r = 240,000; fragment Y, $A^{1\%}/1$ cm, 280 nm = 17.6, M_r = 155,000; fragment D_1, $A^{1\%}/1$ cm, 280 nm = 20.8, M_r = 100,000; fragment E, $A^{1\%}/1$ cm, 280 nm = 10.2, M_r = 50,000. For plasminogen, $A^{1\%}/1$ cm, 280 nm = 16.8, M_r = 92,000.[2,18]

Proteins were radioiodinated by the solid-state lactoperoxidase method.[19] Radioactivity was measured with a AW14-120 gamma counter (Scientific Products, McGraw Park, Ill).

The binding of fibrinogen fragments to murine peritoneal macrophages was examined. Plated macrophages were allowed to equilibrate for one hour at 4 °C and were then washed four times with 1 mL of cold binding medium (10% Earle's balanced salt solution, 50 mmol/L HEPES, 150 mmol/L NaCl, 1% albumin, pH 7.4). After an additional 30 minutes' incubation, the binding medium was replaced with fresh binding medium containing radiolabeled ligand at varying concentrations. After 50 hours (the time required for binding equilibrium; Fig 2) the ligand solution was removed; the cells were washed with cold buffer (50 mmol/L HEPES, 150 mmol/L NaCl, pH 7.4), and allowed to dry. Fibrinogen fragments remain intact after 50 hours under these conditions as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and auto-

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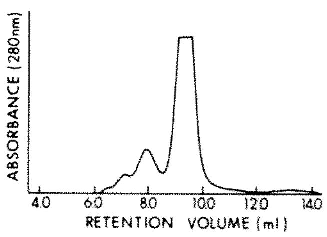

Fig 1. High-performance liquid chromatography of fragment E preparation. Fragment E was obtained by DEAE ion-exchange chromatography and analyzed on a gel filtration column.

radiography of the culture media (data not shown). The cells were then solubilized with 0.3 mL of 0.1% NaOH overnight. The amount of bound ligand was determined by measuring the radioactivity of the solubilized cell solution in a gamma counter. The protein content of each sample was then determined by the Lowry protein assay,[20] as modified by Peterson.[21] This value was converted to numbers of cells by dividing by the average mass of a thioglycollate-stimulated macrophage (80 ng per cell). The amount of bound ligand in each well was expressed as a function of cell number in that well (fmol bound per cell). Well-to-well variations in cell number were compensated for by multiplying this value by the average cell number per well, resulting in an estimate of the total bound ligand in each well.

In one set of experiments, cells were incubated with radiolabeled ligand either alone or in the presence of a 100-fold molar excess of unlabeled ligand to determine nonspecific binding. The amount of radiolabeled ligand bound in the presence of unlabeled ligand (nonspecific binding) was subtracted from the amount bound in the absence of unlabeled ligand (total binding) to determine specific binding. The values for specifically bound ligand, along with values for free ligand at different concentrations, were used to perform Scatchard analyses and obtain estimates of the K_d values for the binding of fibrinogen fragments to macrophage receptors. Because total binding was always less than 1%, the free ligand concentration in each test solution could be estimated from the total amount of ligand originally added to that solution. In another set of experiments, unlabeled fragment D_1 was used to compete with radiolabeled fragments E, X, and Y for binding to macrophages, in order to determine whether these fragments are recognized by the same receptor as fragment D_1.

RESULTS

The binding of fragments D_1 and D_1 dimer to macrophages is shown in Figs 3 and 4 as femtomoles of fragment bound per microgram of cell protein *v* the logarithm of the free fragment concentration. In neither case does the binding curve assume a sigmoidal shape, indicating that no saturation of a finite number of binding sites has been attained.[22-24] Therefore, it is not possible to predict the number of binding sites on macrophages for these fragments. However, because we have demonstrated specific binding, it is valid to derive dissociation constants for such a system.[22-24] The binding data for fragment D_1 are also shown in the form of a Scatchard plot (Fig 5). The Scatchard plot is biphasic, suggesting either the presence of multiple classes of receptors or cooperativity of binding. As an approximation, we assumed the existence of two classes of receptor, a high-affinity receptor for low concentrations of fragment D_1 and a lower-affinity receptor for the higher concentration ranges. The "high-affinity" portion of the Scatchard curve for fragment D_1 binding was more carefully examined by using additional data points for low concentrations of radiolabeled protein (Fig 6). The data fit well to a straight line with a correlation coefficient of -0.99. The corresponding dissociation constant (K_d) was very low, 4.25×10^{-11} mol/L. The possibility that binding to the plastic well accounted for the apparent high-affinity binding was eliminated by performing the study in the absence of cells. In this case, binding of radiolabeled fragments occurred at extremely low levels and was not specific (data not shown).

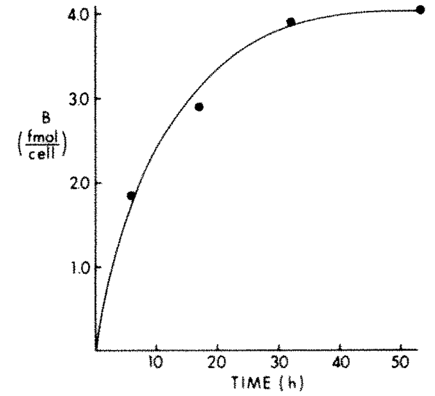

Fig 2. Time dependence of binding of ^{125}I-fragment D_1 to mouse peritoneal macrophages.

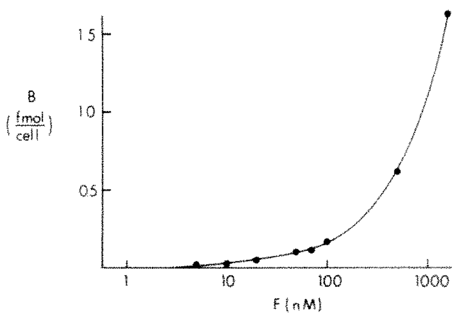

Fig 3. Binding of ^{125}I-fragment D_1 to mouse peritoneal macrophages. Femtomoles of fragment D_1 bound per cell, B, *v* the logarithm of the free fragment D_1 concentration, F.

The binding of fragments E, X, and Y to macrophages in the high-affinity concentration range (1 nmol/L to 100 nmol/L) was also examined and K_d values for fragments D_1

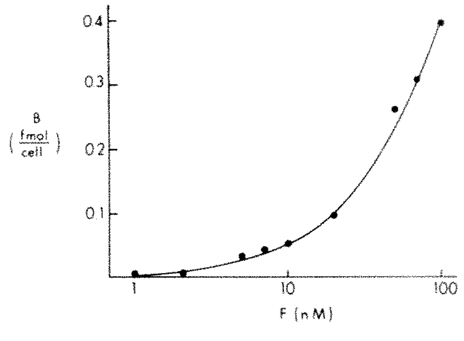

Fig 4. Binding of ^{125}I-fragment D_1 dimer to mouse peritoneal macrophages. Femtomoles of fragment D_1 dimer bound per cell, B, *v* the logarithm of the free fragment D_1 dimer concentration, F.

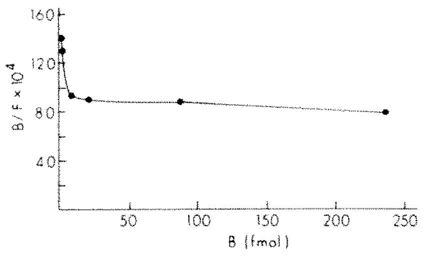

Fig 5. Scatchard analysis of binding of ^{125}I-fragment D_1 to mouse peritoneal macrophages. Data are obtained from Fig 2.

dimer, X, and Y are shown in Table 1. Fragment Y has a slightly higher binding constant and fragments X and D_1 dimer have slightly lower constants than fragment D_1. Fragment E binding (Fig 7) occurred at low levels, less than 10% of those of the other fragments. Moreover, specific binding constituted less than 20% of total binding as opposed to 70% to 80% for the other fragments (data not shown). The higher affinity of binding of fragments X and D_1 dimer as compared with fragment D_1 could be explained if these fragments share the same receptor, since fragments X and D_1 dimer contain two D domains and could therefore be expected to bind more tightly to the cell surface. Again, these K_d values should be regarded only as approximations, due to complexities arising from ligand multivalency and the possible presence of multiple classes of binding sites.

Previous studies[8] have demonstrated that human fibrinogen fragments X and Y, but not E, compete with fragment D_1 for clearance from the circulation of mice, suggesting that the fragment D receptor also mediates the clearance of fragments X and Y.[7,8] We used the in vitro macrophage binding system to examine directly the competition of fragment D_1 for the binding of radiolabeled fragments E, X, and Y (Fig 8). Fragment D_1 competes for the binding of both X and Y in a dose-dependent fashion analogously to the in vivo clearance data. However, fragment D does not compete for the binding of radiolabeled fragment E. These data indicate that all of the fibrinogen degradation products containing the D domain are recognized by the same receptor, and the that D domain contains the recognition site for this receptor system.

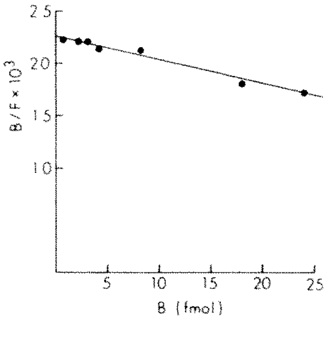

Fig 6. Scatchard analysis of binding of ^{125}I-fragment D_1 to mouse peritoneal macrophages at low ligand concentrations.

Table 1. Dissociation Constants for Binding of Fibrin(ogen) Degradation Products to Macrophages

Fragment	K_d
D	4.25×10^{-11} mol/L
X	2.30×10^{-11} mol/L
Y	7.30×10^{-11} mol/L
D_1 dimer	2.40×10^{-11} mol/L

DISCUSSION

Two of the major functions for biological receptor systems are the removal of specific components from the circulation and participation in the regulation of metabolic pathways. Recent studies in different systems have implicated receptors for plasmin-derived fibrinogen fragments in both roles. The clearance of human fibrinogen fragment D from the circulation of mice is mediated by a specific receptor system in the liver.[7] Furthermore, the clearances of fragments X and Y, but not E, are mediated by the same receptor,[8] suggesting that some determinant in the D domain serves as the receptor recognition site.

Fibrinogen fragments are also involved in the regulation of fibrinogen synthesis by rat hepatocytes.[9-14] Franks et al[12] showed that fragment D, but not fragment E, stimulated fibrinogen synthesis when perfused into rats. However, Richie et al[13] and Sanders and Fuller[14] demonstrated the stimulatory effect with both fragments D and E in their in vitro system. In addition, the latter group showed that the stimulation occurs by way of an indirect feedback pathway, in which fragments D and E elicit the release of hepatocyte-stimulating factor from human blood monocytes and rat Kupffer cells. Hepatocyte-stimulating factor in turn stimulates hepatocytes to synthesize fibrinogen. Mouse peritoneal macrophages appear to participate in a similar regulatory pathway, and supernatants from fragment D-exposed macrophages stimulated the synthesis of fibrinogen, α_1-antitrypsin, and antithrombin III by cultured rat hepatocytes.[25]

The apparent conflict between these studies is further complicated by in vivo observations in the murine system, which suggest that fragment E is not recognized by the D receptor.[7,8] In order to clarify these issues, we applied Scat-

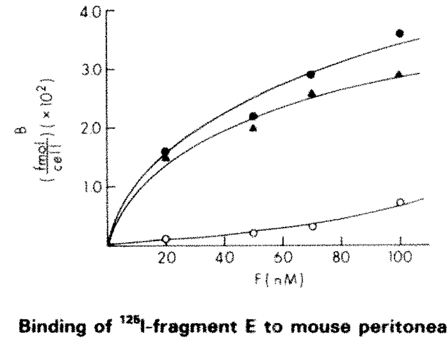

Fig 7. Binding of ^{125}I-fragment E to mouse peritoneal macrophages. Femtomoles of fragment E bound per cell, B, v the free fragment E concentration, F. Specific binding (○) is the difference between total binding (●) and nonspecific binding (▲).

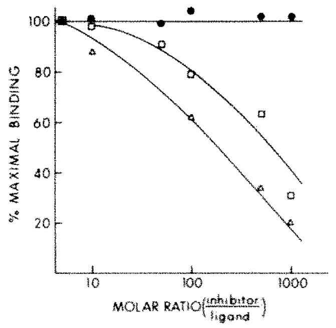

Fig 8. Binding competition experiment. Ligand is radiolabeled fragment E (5 × 10^{-8} mol/L, ●), fragment X (1 × 10^{-8} mol/L, □), or fragment Y (1 × 10^{-8} mol/L, △). Inhibitor is unlabeled fragment D_1 at varying molar ratios to radiolabeled ligand.

chard analyses and competition studies to the in vitro binding of fragments D_1, D_1 dimer, E, X, and Y to cultured mouse peritoneal macrophages. A Scatchard analysis of fragment D_1 binding resulted in a biphasic curve, consistent with, although not strictly indicative of, two classes of receptors. Assuming two receptor classes, the binding at low concentration ranges occurred with an exceptionally low dissociation constant (K_d), 42.5 × 10^{-11} mol/L. The binding of fragments D_1 dimer, X, and Y, all of which contain the D domain, also occur with high affinity. Fragment D_1 also was able to compete for the binding of radiolabeled fragments X and Y, indicating that fragments containing the D domain are recognized by the same receptor. However, fragment D did not compete for the binding of fragment E, indicating that separate receptor systems recognize these fragments. The binding of fragment E to macrophages conforms to the requirements of specific binding. However, so little ligand is bound relative to fragment D binding that uptake of fragments X and Y must occur almost exclusively by the fragment D domain receptor.

During the course of in vivo catabolism of fragments X, Y, D, and E, it is likely that specific uptake mechanisms account for the disappearance of all but fragment E. Clearance of fragment E most likely results from passive glomerular filtration. Consistent with this hypothesis, fragment E is the only one of these fragments that has a mol wt, $M_r \sim 50{,}000$, which is smaller than albumin. Albumin, M_r of ~67,000, is generally thought to represent the renal clearance threshold for a protein.

In light of these observations, it seems unlikely that fragment E would regulate fibrinogen synthesis. Previous studies that showed that fragment E elicits release of hepatocyte-stimulating factor may have used a preparation of fragment E that was contaminated with fragment D. Of interest in this regard is our observation that our fragment E preparation, also obtained by the traditional method of ion-exchange chromatography, displayed a higher mol wt contaminant by HPLC.

Recently, an adenosine diphosphate-dependent platelet receptor system for fibrinogen and von Willebrand factor has been described.[26-28] This receptor, consisting of glycoproteins GP IIb and GP IIIa, specifically recognizes a continuous sequence of 12 amino acids at the carboxyl terminal end of the γ-chain of fibrinogen, in the D domain of the molecule. The isolated glycoprotein complex binds fragment D but not fragment E. Presumably, the GP IIb–GP IIIa complex on the surface of activated platelets interacts with fibrinogen and von Willebrand factor, forming "bridge" structures with the damaged vessel wall and with other platelets.

The macrophage receptor for fragment D_1 described in this study is similar to the platelet GP IIb–GP IIIa receptor, in that it recognizes fragment D but not fragment E. However, unlike the platelet receptor, the macrophage receptor does not recognize the intact fibrinogen molecule.[7] The macrophage receptor does recognize fragment X, which differs from fibrinogen in that the carboxyl terminus of the Aα-chain has been removed. It appears that the receptor recognition site in the D domain for the macrophage receptor is "shielded" by the Aα-chain of intact fibrinogen, while the recognition site for the platelet receptor is exposed in the intact molecule, despite its occurrence in the same domain.

The differences between these two receptor systems are readily understood if one considers their physiologic roles. The GP IIb–GP IIIa receptor on activated platelets uses circulating fibrinogen molecules to facilitate one of the early steps in hemostasis, the formation of the platelet plug. The macrophage receptor, on the other hand, detects fibrinogen degradation products after fibrinolysis has occurred. By way of the release of hepatocyte-stimulating factor from these macrophages, the synthesis of fibrinogen is increased and circulating levels are returned to normal.

REFERENCES

1. Nussenzweig V, Seligmann M, Pelmont J, Grabar P: Les produits de degradation du fibrinogene humain par la plasmine. Ann Inst Pasteur Paris 100:377, 1961

2. Marder VJ, Shulman NR, Carroll WR: High molecular weight derivatives of human fibrinogen produced by plasmin. J Biol Chem 244:2111, 1969

3. Pizzo SV, Schwartz ML, Hill RL, McKee PA: The effect of plasmin on the subunit structure of human fibrinogen. J Biol Chem 247:636, 1972

4. Pizzo SV, Taylor LM, Schwartz ML, Hill RL, McKee PA: Subunit structure of fragment D from fibrinogen and cross-linked fibrin. J Biol Chem 248:4584, 1973

5. Haverkate F, Timan G: Protective effect of calcium in the plasmin degradation of fibrinogen and fibrin fragments D. Thromb Res 10:803, 1977

6. Catanzaro A, Hathaway G, Strathern J, Edgington T: Structure and in vivo behavior of human fibrinogen fragment-D′. Proc Soc Exp Biol Med 139:1401, 1972

7. Pasqua JJ, Pizzo SV: The clearance of human fibrinogen fragments D_1, D_2, D_3, and fibrin fragment D_1 dimer in mice. Biochim Biophys Acta 718:177, 1982

8. Pasqua JJ, Pizzo SV: The clearance of human fibrinogen fragments X and Y in mice: A process mediated by the fragment D receptor. Thromb Haemost 49:78, 1983

9. Barnhardt MI, Cress DC, Noonan SM, Walsh RT: Influence of fibrinolytic products on hepatic release and synthesis of fibrinogen. Thromb Diath Haemorrh 29:63, 1973

10. Bocci V, Pacini A: Factors regulating plasma protein synthesis. II. Influence of fibrinolytic products on plasma fibrinogen concentration. Thromb Diath Haemorrh 29:63, 1973

11. Bocci V, Conti T, Muscellata M, Pacini A, Pessina GP: Factors regulating plasma protein synthesis. IV. Influence of fragments D and E on plasma fibrinogen concentration. Thromb Diath Haemmorh 31:395, 1974

12. Franks JJ, Kirsch RE, Frith OC, Purves LR, Franks JA, Mason P, Saunders SJ: Effects of fibrinolytic products D and E on albumin synthesis in the rat. J Clin Invest 67:574, 1981

13. Ritchie DG, Levy BA, Adams MA, Fuller GM: Regulation of fibrinogen synthesis by plasmin-derived fragments of fibrinogen and fibrin: An indirect feedback pathway. Proc Nat Acad Sci USA 79:1530, 1982

14. Sanders KD, Fuller GM: Kupffer cell regulation of fibrinogen synthesis in hepatocytes. Thromb Res 32:133, 1983

15. Chen JP, Shurley HM, Vickroy MF: A facile separation of fragments D and E from the fibrinogen/fibrin degradation products of three mammalian species. Biochem Biophys Res Commun 61:66, 1974

16. Deutsch DG, Mertz ET: Plasminogen purification from human plasma by affinity chromatography. Science 170:1095, 1970

17. Brockway WJ, Castellino FJ: Measurement of the binding of antifibrinolytic amino acids to various plasminogens. Arch Biochem Biophys 151:194, 1972

18. Sjoholm I, Wiman B, Wallen P: Studies on the conformational change of plasminogen induced during activation to plasmin and by 6-amino-hexanoic acid. Eur J Biochem 39:471, 1973

19. David GS, Reisfeld RA: Protein iodination with solid-state lactoperoxidase. Biochemistry 13:1014, 1974

20. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

21. Peterson GL: Review of the Folin phenol protein quantitation method of Lowry, Rosebrough, Farr, and Randall. Anal Biochem 100:201, 1979

22. Klotz IM, Hunston DL: Protein interactions with small molecules: Relationships between stoichiometric binding constants, site binding constants, and empirical binding parameter. J Biol Chem 250:3001, 1975

23. Klotz IM, Hunston DL: Protein affinities for small molecules: Conceptions and misconceptions. Arch Biochem Biophys 193:314, 1979

24. Klotz IM: Numbers of receptor sites from Scatchard graphs: Facts and fantasies. Science 217:1247, 1982

25. Hoffman M, Fuchs HE, Pizzo SV: The macrophage-mediated regulation of hepatocyte synthesis of antithrombin III and α_1-proteinase inhibitor. Thromb Res (in press)

26. Nachman RL, Leung LLK, Kloczewiak M, Hawiger J: Complex formation of platelet membrane glycoproteins IIb and IIIa with the fibrinogen D domain. J Biol Chem 259:8584, 1984

27. Timmons S, Kloczewiak M, Hawiger J: ADP-dependent common receptor mechanism for binding of von Willebrand factor and fibrinogen to human platelets. PNAS 81:4935, 1984

28. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J: Platelet receptor recognition site on human fibrinogen synthesis and structure-function relationship of peptides corresponding to the carboxyl-terminal segment of the γ chain. Biochem 23:1767, 1984

Hemostatic Plug Formation in Normal and von Willebrand Pigs: The Effect of the Administration of Cryoprecipitate and a Monoclonal Antibody to Willebrand Factor

By Y. Sawada, D.N. Fass, J.A. Katzmann, R.C. Bahn, and E.J.W. Bowie

Hemostatic plug (HP) formation was investigated in the ear bleeding time incision in normal and von Willebrand pigs. HP volume was calculated by integrating the areas of serial sections. In normal pigs (n = 11), platelets immediately formed a layer on the surface of the cut channel. Platelet aggregates formed at the ends of transected vessels and gradually enlarged. Finally, all transected vessels were occluded by HP and bleeding stopped. In contrast, large HPs were formed in the incision in von Willebrand's disease (vWD) pigs (n = 4); these HPs did not cover the ends of the transected vessels, which continued to bleed, allowing the formation of large hemostatically ineffective platelet aggregates in the incision. Canals traversed these HPs, and bleeding from the open vessels may have continued through them. After infusion of cryoprecipitate into a vWD pig, the bleeding time shortened, and the morphological findings of the HPs were similar to those of normal pigs. In normal pigs (n = 3) infused with an anti-Willebrand factor monoclonal antibody, which prolonged the bleeding time, a large HP formed in the incision, similar to that observed in the vWD pig. The volume of the normal and vWD HPs increased with time. These in vivo findings suggest that Willebrand factor is involved in the localization of the HP to the damaged vessel and may also play a role in platelet-platelet interaction. A computerized morphometric technique was used for measuring the volume of the hemostatic plugs and the distance of sequential points on the perimeter of the HP from the center of selected bleeding vessels.

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MORPHOLOGICAL STUDIES of the bleeding time incision by light and electron microscopy have described the hemostatic plug in normal hemostasis. These investigations have been done in normal individuals,[1] as well as in humans with hemophilia A, factor VII deficiency, von Willebrand's disease, macroglobulinemia, and thrombasthenia[2] and in dogs with factor VII deficiency and hemophilia A and B.[3]

There have been few extensive investigations during the process of hemostasis.[4-6] Wester et al[4] investigated hemostatic plug formation in human skin wounds and suggested that aggregation of platelets stopped the bleeding and that fibrin played a secondary role in primary hemostasis.

To investigate the quantitative changes of the platelets in hemostasis, Borchgrevink[7] and Johnson et al[8] measured the platelet retention in bleeding time incisions by counting platelets in the venous blood and in the blood flowing from the incision. Recently, Kelton and Carter[9] reported quantitative studies evaluating the platelet component of the hemostatic plugs in the bleeding time incision in rabbits' ears.

The pathogenesis of von Willebrand's disease has been studied intensively,[10,11] but only two morphological investigations of the bleeding time incisions have been done[2,12] in patients with von Willebrand's disease.

We have maintained a colony of pigs with von Willebrand's disease, similar to the severe homozygous type of human von Willebrand's disease.[13,14] The availability of this animal model has allowed us to investigate the bleeding time incisions morphologically. In this study, we describe a time sequence study of the formation of the hemostatic plug in normal and von Willebrand pigs by light microscopy, using a new computerized method to calculate the volume of the hemostatic plugs formed in the channel of the bleeding time incision. Using this method, the morphology of platelet blood vessel interaction is described, as well as quantitative changes in hemostatic plug formation. In addition, we describe the effect of giving cryoprecipitate to a von Willebrand pig and a monoclonal antibody against Willebrand factor to a normal pig.

MATERIALS AND METHODS

Definition of Terms

In this article, von Willebrand factor refers to the multimeric protein that is absent, decreased, or abnormal in von Willebrand's disease. Factor VIII refers to factor VIII coagulant activity measured by the degree of correction of the activated partial thromboplastin time of human hemophilia A plasma.[15] Values above 1% of normal can be reliably measured for this factor. One percent of pig factor VIII activity is equivalent to approximately 8% human factor VIII.[16]

Von Willebrand antigen (vWF-Ag) refers to the antigenic activity detected by a rabbit antibody to purified von Willebrand factor[17] measured by electroimmunoassay.[18] We calibrate this assay to a lower limit of 3%.

Ristocetin cofactor (R.Co.) refers to the activity of von Willebrand factor that allows ristocetin to induce platelet aggregation. This activity is missing or reduced in the plasma in von Willebrand's disease. R.Co. is measured by the degree of aggregation of washed gel-filtered human platelets using Olson's modification[19] of Weiss' method.[20] The standard curve for this assay extends from 6.25% to 50%. Values as low as 3% were measured by extrapolation.

The von Willebrand Pig Colony

For several years, we have maintained a breeding colony of pigs with von Willebrand's disease.[16] These animals share the impairment of primary hemostasis and other hemostatic abnormalities of the severe form of the disease in humans.[21,22] The animals have a serious bleeding tendency,[13,16] which is transmitted as an autosomal recessive characteristic.[23,24] The abnormal tests of hemostasis include prolongation of the bleeding time, almost complete absence

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of von Willebrand antigen (0.25% of normal), and the lack of R.Co. in the plasma.

Hemostatic Studies in Normal and von Willebrand Pigs

Eleven normal mixed-breed pigs (Duroc, Yorkshire, and Hampshire), weighing 36 to 45 kg, were used for the study of normal morphology and two for immunoperoxidase staining to investigate the localization of von Willebrand antigen. Three normal pigs, weighing 7.2 to 8.1 kg, were used for the study with monoclonal antibodies.

Four von Willebrand pigs, weighing 41 to 45 kg, were used. The factor VIII levels varied from 32 to 44 U/dl and the levels of von Willebrand antigen and R.Co. were <3 U/dL. None of the normal or von Willebrand pigs had ever been previously infused with whole blood or any blood products.

Cryoprecipitate Infusion into von Willebrand Pig

Cryoprecipitate was made from normal pig plasma by the method of Schlichter et al.[25] Cryoprecipitate (factor VIII 2350 U, vWF-Ag 1650 U, R.Co. 1800 U; 973 mL) was infused into a vein on the posterior surface of the ear. The blood samples were taken from the antecubital vein on the foreleg.

Bleeding Time

The ear bleeding time test was done by Mertz'[26] modification of the immersion method of Doettl and Ripke.[27] The pig was placed on its back on a wooden cradle, restrained by a rope laced across its body, and allowed to lie quietly for half an hour. The ear was cleaned and shaved, and an incision was made through the thickness of the ear near the apex, using a No. 11 Bard-Parker blade 3 mm thick. The width of the incision was controlled by inserting the blade to a depth of 5 mm. The ear was immediately placed in a beaker of isotonic saline that had been warmed to 37 °C, and the time of bleeding was measured. The bleeding time was the time that elapsed between the puncture and the cessation of any visible flow of blood from the incision. Care was taken to avoid small veins on the posterior surface of the ear.

At different intervals after the bleeding time started, the incision, together with the surrounding area, was excised through the complete thickness of the ear, using a circular biopsy punch 9 mm in diameter. The biopsy procedure took less than one second.

Tissue Preparation

The biopsy specimens for histologic studies were immediately immersed in Orth's solution for fixation. After two-day fixation, the tissues were embedded in paraffin. Serial sections (6 μ thick) were made perpendicular to the surface of the skin and the incision. Every tenth section was stained with Giemsa stain.

The biopsy specimens for immunohistochemical studies were immediately frozen in liquid nitrogen and stored at −70 °C until used.

Monoclonal Antibodies

The monoclonal antibodies to von Willebrand factor that were used in these studies are fully described by Katzmann et al.[28] The antibodies to von Willebrand factor were originally identified by a radioimmunoassay based on the ability of immobilized hybridoma antibodies to bind porcine Willebrand factor as detected by the subsequent binding of an affinity-purified polyclonal rabbit anti-Willebrand factor labeled with ^{125}I.

For each hybridoma line, ascites fluids were obtained in the absence of anticoagulant, and the harvested fluids were clarified by centrifugation. The fluids for an individual hybridoma were pooled, aliquoted, and frozen. The antibodies were titrated by determining the dilution of ascites fluid at which 50% binding of von Willebrand factor occurred when compared with binding levels at antibody saturation. In this way, a relative potency could be given and the antibodies could be compared for their effect in the different tests.

Monoclonal Antibody Injection into Normal Pig

One milliliter of monoclonal antibody W1-8 was infused through the vein of the posterior surface of the ear. Preinfusion and postinfusion bleeding times were measured in the ear through which monoclonal antibody was infused. On the other ear, four bleeding time incisions were made simultaneously five minutes after the infusion, and each incision was biopsied either at 1, 2, 7, and 15 minutes after incisions had been made, respectively.

Morphometric Studies

Paraffin sections 6 μ in thickness were made of the entire incised lesion. The plane of sectioning was oriented perpendicular to the plane of the incision and perpendicular to the plane of the skin. Every tenth section was stained with Giemsa stain. Multiple photomicrographs (× 155) were taken of each plane so that a complete photomontage of the incision could be reconstructed.

On each photomontage, cross sections of the hemostatic plugs were outlined and numbered. The area of each hemostatic plug was measured with a computer-linked planimeter (HIPAD; Houston Instrument, Austin, Tex). The number of animals, the total number of histologic planes, and average number of histologic planes studies are recorded in Table 1.

Recorded in Table 2 for each experimental procedure and for each time interval are the number of plug cross sections, the cross-sectional area of plug, the total cross-sectional area of plug, and the total volume of plug. These quantities are expressed as average values per experimental animal. The average cross-sectional area of plug is expressed as empirical relative units of area (Table 2). The average total cross-sectional area of plug was calculated by multiplying the average cross-sectional area of plug per animal by the average number of plug cross sections per animal. The average total volume of plug per animal was calculated by multiplying the average total cross-sectional area of plug per animal by the average interplanar distance. Thus, the total volume of plug per animal is expressed as empirical relative units of volume (Table 2).

The digitizing pad (HIPAD, Houston Instrument) was also used in a few selected animals to measure the distance from the center of small lacerated arteries to points on the perimeters of adjacent hemostatic plugs. The number of points collected in each photomontage ranged from 61 to 441, with an average of 220. The distances were recorded as relative linear units.

RESULTS

The Bleeding Time Incision in Relation to the Morphology of the Pig Ear

The pig ear consists of epidermis, superficial layer of the dermis, deep layer of the dermis, and cartilage. The histologic stucture of the anterior surface is similar to that of the posterior surface, although the former has more hair follicles than the latter.

The dermis is composed of a network of collagen, reticular and elastic fibers, hair follicles, sweat glands, adipose tissue, and vessels. Networks of vessels are present, especially in the capillaries of the dermal papillae that form loops. Small arteries and veins exist in the deep layer close to the cartilage.

Table 1. Results of Morphometric Studies

Experiment				
Group	Time	No. of Animals	Total No. of Histologic Planes	Average No. of Histologic Planes per Animal
Normal	10	3	45	15
	30	5	120	24
	60	6	164	27
	120	6	197	33
	180	2	88	44
	Total	22	624	28.4
Normal; monoclonal antibody	60	2	67	34
	120	2	26	13
	420	1	26	26
	Total	5	119	23.8
von Willebrand	10	3	55	18
	30	2	54	27
	60	2	46	23
	120	2	40	20
	180	2	71	35
	420	3	104	35
	900	2	54	27
	Total	16	424	26.5
von Willebrand; cryoprecipitate	10	1	8	8
	30	1	26	26
	60	1	24	24
	120	1	11	11
	180	1	16	16
	420	1	21	21
	Total	6	106	17.6

Table 2. Results of Morphometric Studies

Experiment		Average per Animal			
Group	Time	No. of Plug Cross Sections	Cross-sectional Area of Plug*	Total Cross-sectional Area of Plug*	Total Volume of Plug*
Normal	10	8	4.5	36	0.5
	30	50	9.1	455	109
	60	265	15.8	4,187	138
	120	286	28.7	8,208	263
	180	268	90.0	24,120	1,061
Normal; monoclonal antibody	60	360	5.4	1,944	66
	120	213	56.4	12,013	156
	420	244	60.9	14,860	386
von Willebrand	10	15	3.1	47	0.8
	30	181	4.2	760	20
	60	410	9.0	3,690	85
	120	269	28.9	7,774	155
	180	634	18.9	11,983	419
	420	479	123.4	59,348	2,077
	900	308	121.4	37,391	1,010
von Willebrand; cryoprecipitate	10	18	3.5	64	0.5
	30	131	5.1	668	174
	60	219	10.8	2,365	568
	120	102	6.5	663	7
	180	278	44.3	12,315	197
	420	419	74.0	31,006	651

*Relative empirical units.

The cut channel was made through the whole thickness of the ear. Networks of the capillaries, arterioles, venules, and small arteries and veins were transected. The largest diameter of the transected small arteries was about 280 μ.

The Morphology of the Hemostatic Plug in Normal Pigs

Five experiments were performed in normal pigs and biopsies were taken at 10, 30, 60, 120, and 180 seconds.

At ten seconds after the incision. In the channel, very small hemostatic plugs ($\leq 20\ \mu$) were already present. They were adhering to the ends of the transected vessels or connective tissue, but others were observed free of any attachment. In two of the five experiments, no hemostatic plugs were seen. This did not necessarily mean that there were no plugs because plugs were small and only every tenth section was examined. The appearance of these plugs was homogeneous and contained neither red cells nor leukocytes. At the end of the transected vessels, aggregated platelets were observed.

The margin of the bleeding time incision was covered with a layer of platelets. This layer of platelets and the aggregated platelets were further identified by PAP staining,[29] using an antibody to pig Willebrand factor.

At 30 seconds after the incision. At this point, more hemostatic plugs appeared. Most of the hemostatic plugs were found associated with vessels and about 20% were unattached to either the transected vessels or connective tissue. At the cut surface of the small arteries, hemostatic plugs were formed, but at this stage, they were too small to cover the entire cut area (Fig 1).

At 60 seconds after the incision. The ends of some small arteries were completely covered with hemostatic plugs (Fig 2), but some were not. At this stage, some hemostatic plugs formed near the transected vessels bridged the bleeding time incision and were attached to the connective tissue in the superficial layer of the dermis. Hemostatic plugs attached to the incised surface of the epidermis were observed.

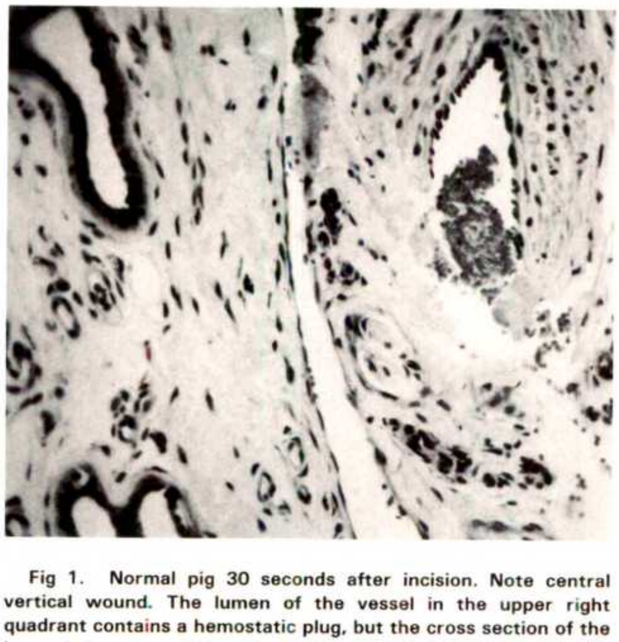

Fig 1. Normal pig 30 seconds after incision. Note central vertical wound. The lumen of the vessel in the upper right quadrant contains a hemostatic plug, but the cross section of the lumen is less than 50% occluded (Giemsa, original magnification × 64; current magnification ×56).

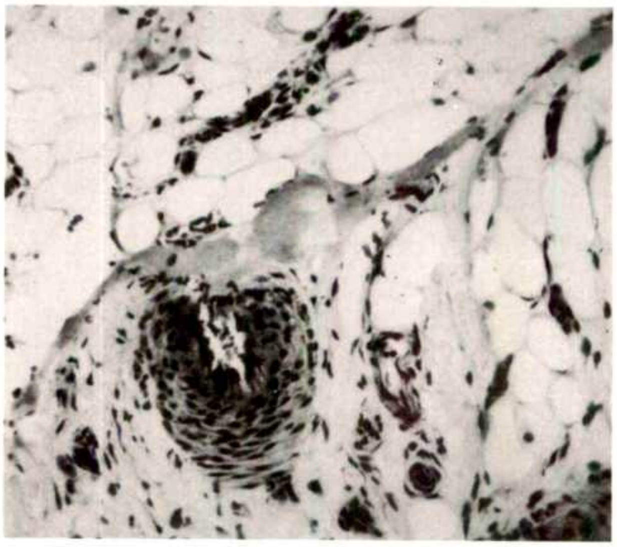

Fig 2. Normal pig 60 seconds after incision. Note wound extending from the right upper corner of photo to the lumen of a lacerated small artery. The arterial mural defect is completely covered by hemostatic plug, which in addition, occupies the very proximal portions of the experimental wound (Giemsa, original magnification ×64; current magnification ×56).

Material suggesting fibrin fibers was observed (Giemsa stain), especially along the cut surface of the connective tissue. This material occasionally seemed to trap red blood cells and leukocytes and could be seen around the hemostatic plugs.

In general, larger hemostatic plugs were formed in the deep layer of the dermis, where a number of transected small arteries and veins were seen. Hemostatic plugs attached to the cartilage were also observed.

At 120 seconds after the incision. There was a great deal of variability in the size of the hemostatic plugs. As was in the case at 60 seconds, these large hemostatic plugs tended mainly to be adhering to small arteries or veins. In the channel of the superficial layer where papillary networks were observed, some hemostatic plugs appeared to occlude the channel. The appearance of the hemostatic plugs was occasionally different from those formed at the earlier stages. Large plugs seemed to be composed of several small plugs. Between plugs there was some material suggesting fibrin and ballooned platelets.

The Morphology of the Hemastatic Plug in von Willebrand Pigs

Three experiments were performed in von Willebrand pigs. The hemostatic plugs were found in the incision, and there was no difference between von Willebrand pigs and normal pigs at ten seconds after the incision. The structure of the cut channel was similar to that of normal pigs at 30 seconds. The hemostatic plugs were found adhering to the

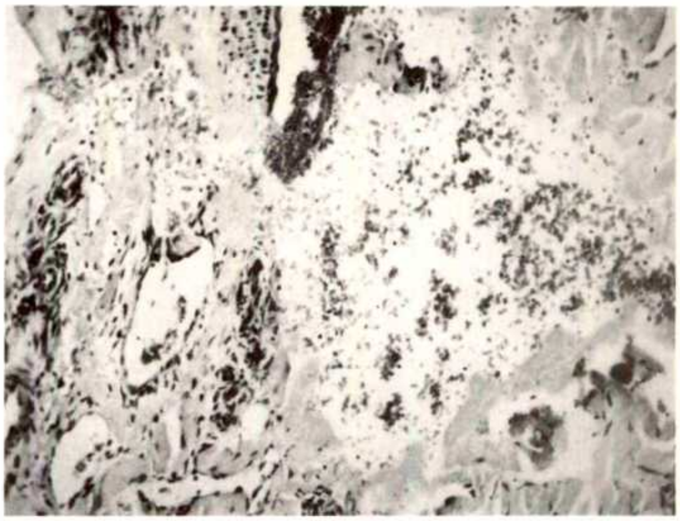

Fig 3. Von Willebrand pig two minutes after incision. Note cavity of wound extending from the left upper corner to the right central portion of the photo. The left portion of the channel contains hemostatic plugs, but no vessels are completely occluded (Giemsa, original magnification ×40; current magnification ×34).

surface of the cut incision, and some were adhering to the ends of the transected vessels. At 60 seconds, while the cut ends of small arteries were completely covered with the hemostatic plug in normal pigs, the arteries were only partially covered in von Willebrand pigs, despite the fact that the volume of the hemostatic plugs was the same as that of normal pigs.

At two minutes, hemostatic plugs were adhering to the ends of the transected vessels or to the surface of the cut channel, but no arteries were completely occluded (Fig 3).

At three minutes, however, some of the connective tissue area in the channel was completely covered with large hemostatic plugs, but still the ends of the transected vessels were not covered. At seven minutes, the volume of the hemostatic plugs in the incision continued to enlarge, resulting in the covering of the whole inner surface of the incision except the ends of the transected small arteries (Fig 4).

At 15 minutes, small arteries were again not covered with the hemostatic plugs, which completely filled the incision. An irregular canal filled with red blood cells and leukocytes made a track through the hemostatic plugs, as if bleeding was continuing through it (Fig 5).

The Morphology of the Hemostatic Plug After Cryoprecipitate Infusion into a von Willebrand Pig

Five minutes after cryoprecipitate infusion, bleeding time tests were done and had shortened to six and eight minutes on two occasions. Seven bleeding time incisions were made simultaneously for morphological study and they were biopsied at ten and 30 seconds and at 1, 2, 3, 7, and 15 minutes. Within ten seconds, small hemostatic plugs were already observed. At 30 seconds, hemostatic plugs were found adhering to the ends of the transected vessels, and at one minute, some vessels were completely occluded (Fig 6). At three minutes, relatively large hemostatic plugs were found attaching to the transected small arteries. The appearance of the hemostatic plug was homogeneous. At seven minutes, the transected vessels were covered with the hemostatic plugs and no canal-like structure was observed (Fig 7). At 15 minutes, when bleeding stopped in the incision, some hemo-

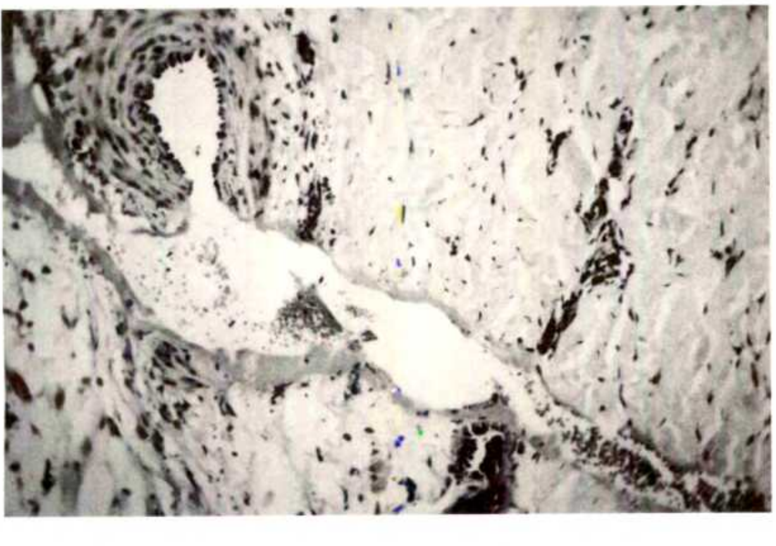

Fig 4. Von Willebrand pig three minutes after incision. Note the cavity of the incision extending from the left upper corner to the right lower corner of the photo. A lacerated artery is present in the left upper quadrant. Platelets line the walls of the incision at a distance from the lacerated small artery, but the mural arterial laceration is not occluded (Giemsa, original magnification ×40; current magnification ×34).

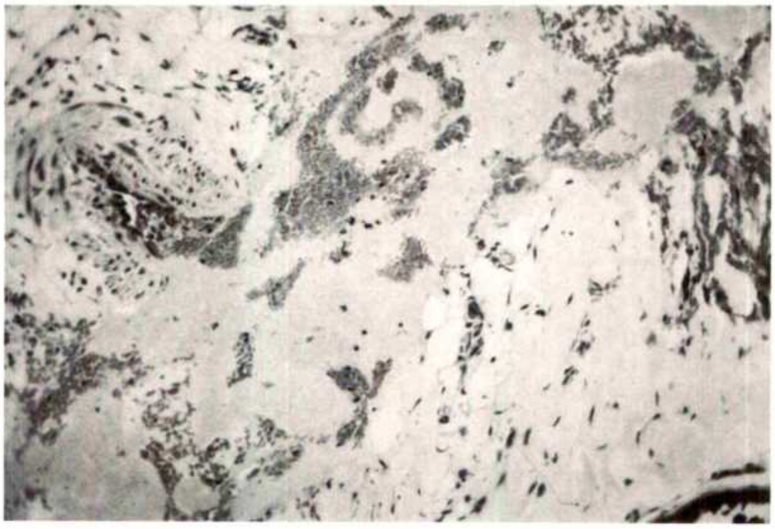

Fig 5. Von Willebrand pig 15 minutes after incision. Note wide channel of laceration extending from left lower to right upper corner of photo. A lacerated artery is present in the left upper quadrant. Hemostatic plug completely distends the incision. However, the mural arterial laceration contains only erythrocytes apparently contiguous with plexiform channels throughout the hemostatic plug (Giemsa, original magnification ×64; current magnification ×54).

static plugs developed fibers like fibrin and red blood cells and leukocytes appeared to be interlaced by this fibrous material.

The Effect of Infusing Monoclonal Antibody W1-8 on the Bleeding Time and the Morphology of the Hemostatic Plug

Ear bleeding time. A normal pig was injected with 0.4 mL of anti-Willebrand monoclonal antibody W1-8, which can prolong the bleeding time to 15 minutes and has a more pronounced effect on the bleeding time than our other monoclonal antibodies.[30] There were no changes in the levels of factor VIII, von Willebrand antigen, and R.Co.

Morphological changes in the bleeding time incisions. Biopsy specimens were obtained while bleeding was occurring at one minute and two minutes. At seven minutes, bleeding almost stopped. At 15 minutes, bleeding was not observed.

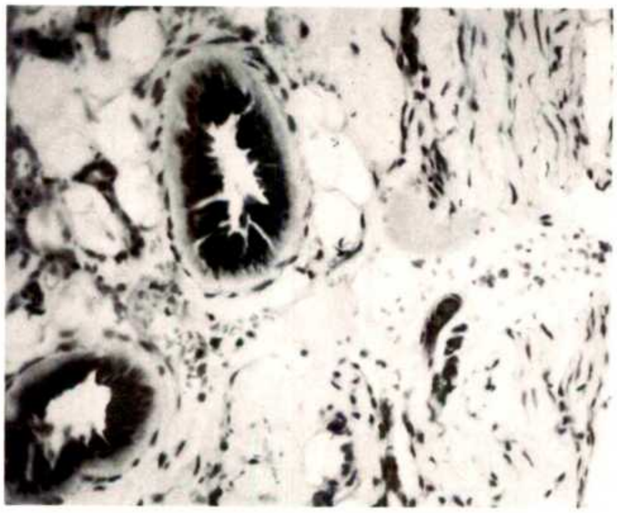

Fig 6. Von Willebrand pig after administration of cryoprecipitate, 30 seconds after incision. Hemostatic plugs are adherent to some small transected vessels (Giemsa, original magnification ×64; current magnification ×54).

Morphology of the hemostatic plugs at one minute was basically similar to that of the normal study observed at one minute after incision had been made. Some hemostatic plugs were formed at the ends of the transected vessels and some were adhering to the surface of the cut channel. Larger hemostatic plugs were found close to the transected vessels as seen in a normal pig. At seven minutes, biopsies were done while the incision was oozing; huge hemostatic plugs were found and the ends of the small arteries were not covered with the hemostatic plugs (Fig 8). Canal-like structures that were packed with red blood cells and leukocytes could be seen in the large hemostatic plugs, suggesting that bleeding continued through this canal. This structure is consistent with that previously described in the von Willebrand pig. At 15 minutes, huge hemostatic plugs were seen (Fig 9) and the appearance was different from that at 1, 2, and 7 minutes. Granular appearance due to individual platelets were lost and a fibrinous network appeared. In the canal-like structure, red blood cells and leukocytes seemed to be interlaced by the fibrinous tissue.

Localization of the infused monoclonal antibody. PAP staining was performed in the cryostat sections to investigate the localization of the infused monoclonal antibody W1-8 in the tissue including the bleeding time incision. W1-8, mouse antiporcine von Willebrand factor, linked to porcine von Willebrand factor was detected by adding rabbit antimouse antibody and, subsequently, mouse PAP. W1-8 was localized to the hemostatic plugs composed of platelets, individual platelets attached to the surface of the cut channel or fibrous tissue, and endothelial cells of the vessel. Platelets showed more intense staining than did endothelial cells. One minute after the bleeding time incision had been made, that is, six minutes after monoclonal antibody infusion, all above-mentioned components were positive for W1-8 and there was no increase in the reaction subsequently at 2, 7, and 15 minutes.

Morphometric Studies

The number of animals studied at various time intervals, the total number of histologic planes, and the average

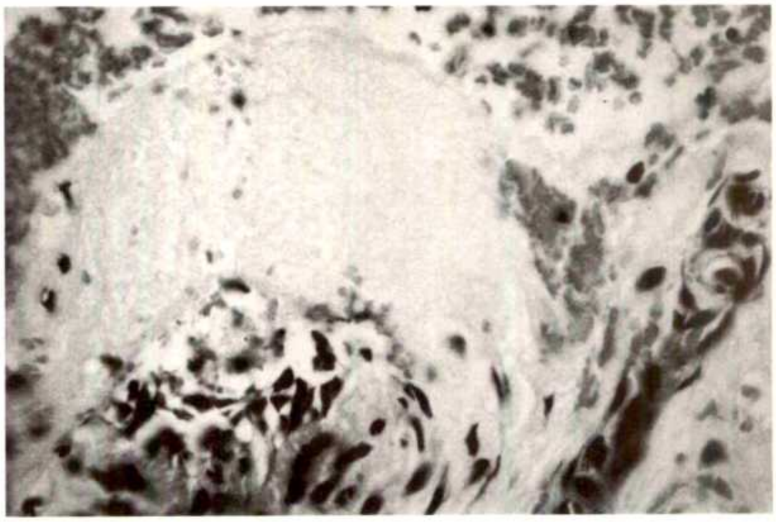

Fig 7. Von Willebrand pig after administration of cryoprecipitate, seven minutes after incision. The mural arterial laceration is completely occluded by a hemostatic plug. The plug is homogenous and does not show any plexiform channels (Giemsa, original magnification ×160; current magnification ×134).

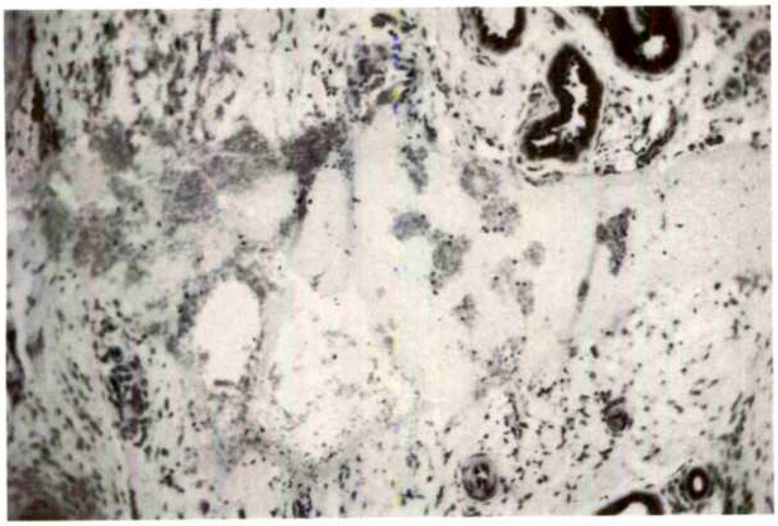

Fig 8. Normal pig after administration of monoclonal antibodies, 15 minutes after incision. Note horizontal channel of laceration distended by hemostatic plug showing plexiform pattern of channels containing erythrocytes. Lacerated wall of artery (upper middle) is not occluded (Giemsa, original magnification ×40; current magnification ×34).

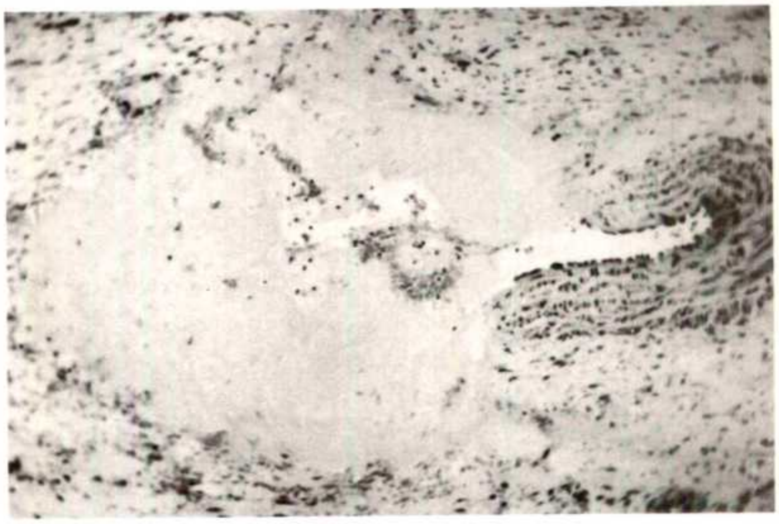

Fig 9. Normal pig after administration of monoclonal antibody, 15 minutes after incision. Lacerated wall of artery (right) not occluded, but communicates with small plexiform channel of hemostatic plug (Giemsa, original magnification ×40; current magnification ×34).

number of histologic planes per animal are recorded in Table 1. The average number of hemostatic plug cross sections, the average cross-sectional area of a hemostatic plug, the average relative total cross-sectional area of hemostatic plugs, and the average relative total volume of hemostatic plugs are recorded in Table 2. Table 2 demonstrates that in all experimental groups, as time increased, the hemostatic plugs increased with respect to numbers, cross-sectional areas, and total volume. Figure 10 shows the growth of total volume of hemostatic plugs from 0 to 900 seconds in experiments involving normal pigs, von Willebrand disease pigs, normal pigs given anti-Willebrand factor monoclonal antibody, and pigs with von Willebrand's disease given cryoprecipitate. These values varied considerably among individual animals. The average values extend over several orders of magnitude (Table 2, Fig 10). The graphs of the average total volumes of hemostatic plugs (Fig 10) demonstrates essentially no difference among the various experimental groups of animals.

While at any one time period the total volume of hemostatic plugs was essentially similar among all experimental groups, bleeding time values indicated that the normal clotting process terminated promptly. Moreover, morphological studies of normal animals demonstrates prompt and complete sealing of small arteries (Figs 1 and 2) as opposed to incomplete plugging and plexiform channels among von Willebrand disease pigs (Figs 3, 4, and 5).

The implication of these observations is that the hemostatic plug material, while of about the same total volume as in normal pigs, is distributed differently about bleeding sites of von Willebrand disease pigs. To test this hypothesis, in selected cases (Figs 3 through 9), the distance of points about the perimeters of hemostatic plugs from the center of a small lacerated artery was measured with the computerized digitizing pad. It should be noted that these quantitative data, based on measurements of the previously described photomontages, extend the above qualitative histologic observations to contiguous areas of the experimental wound, which are about five to ten times greater than the single microscopic fields shown in Figs 1 through 9.

The quantitative results are shown in Figs 11 and 12 and summarized in Table 3. Figure 11 shows that in the normal pig at 120 seconds, the hemostatic plugs are in the immediate vicinity of the arterial laceration and that essentially all the plugs are less than 6 linear units' distance from the lacerated small artery. These findings were termed the normal pattern.

Table 3. Studies of the Distance of Points on the Perimeters of Hemostatic Plugs from the Center of a Small Lacerated Artery

State	Time (s)	Maximum Distance (units)	Percentage of Points >5 Units	Reference Figures
Normal	120	6	1	1,2,11,12
vW	120	12	55	3,11*
vW	420	18	63	4,11*
vW	900	27	93	5,11,12*
vW + CP	120	4	0	6,7,12*
Normal + MC	420	23	91	8,9,12*

vW, von Willebrand's disease; CP, cryoprecipitate; MC, monoclonal antibody.

*Photomicrographs and measurements in same animal.

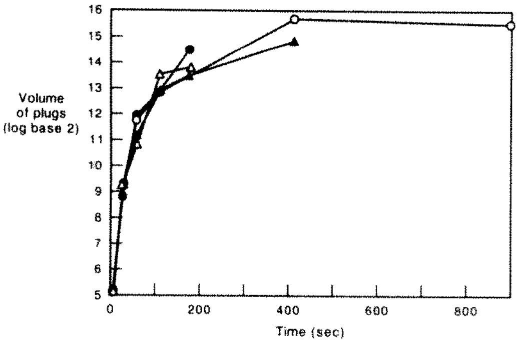

Fig 10. **Comparison of volume of hemostatic plug in normal pig (●), von Willebrand pig (O), von Willebrand pigs given cryoprecipitate (Δ), and normal pigs given anti-Willebrand factor monoclonal antibody (▲). No difference in the pattern of growth of hemostatic plug is demonstrable among these treatments.**

In contrast, in pigs with von Willebrand's disease, at all time intervals, hemostatic plugs were generally not in the immediate vicinity of the arterial laceration. More than half of the perimeter points were greater than 5 units' distance from the lacerated artery and the maximum distance at 900 seconds is 27 units. These results were termed the bleeding pattern.

Figure 12 and Table 3 indicate that administration of cryoprecipitate changed the bleeding pattern of the pig with von Willebrand's disease to the normal pattern. On the other hand, the administration of an anti-Willebrand factor monoclonal antibody to the normal pig transformed the normal pattern to a bleeding pattern.

DISCUSSION

It has been suggested that von Willebrand factor plays an important role in platelet–platelet interaction.[31] The antibiotic ristocetin induces platelet aggregation in normal plate-

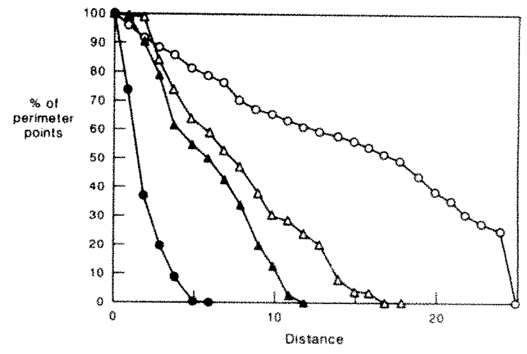

Fig 11. **The proportion of points on the surface of hemostatic plugs as a function of distance from the center of a lacerated artery in a normal pig at 120 seconds (●) and von Willebrand pigs at 120 (▲), 340 (Δ), and 900 seconds (O). The hemostatic plugs in the normal pig are found occluding the laceration and extending in the proximal regions of the lacerated vessel. The hemostatic plugs of the von Willebrand pig are not found occluding the laceration and extend to relatively far distances from the lacerated vessel.**

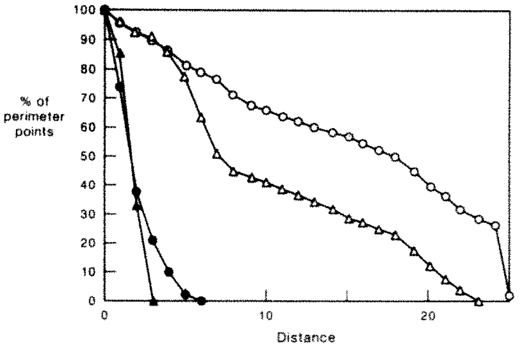

Fig 12. The proportion of points on the surface of hemostatic plugs as a function of the distance from the center of a lacerated artery in a normal pig at 120 seconds (●), a von Willebrand pig given cryoprecipitate at 120 seconds (▲), a normal pig given anti-Willebrand factor monoclonal antibody at 420 seconds (△), and a von Willebrand pig at 900 seconds (○). Cryoprecipitate induces a "normal" pattern in a von Willebrand pig. Anti-Willebrand monoclonal antibody induced a von Willebrand pattern in a normal pig.

let-rich plasma.[32] In this reaction, platelet glycoprotein Ib (GP Ib) functions as a receptor for von Willebrand factor.[33,34] Ristocetin-induced platelet aggregation is absent in the Bernard-Soulier syndrome, in which GP Ib is missing from the platelets and is inhibited by adding monoclonal antibodies against either GP Ib[33,34] or von Willebrand factor.[28,35] Recently, it has also been shown that GP IIb/IIIa appears to be a receptor for von Willebrand factor after the addition of thrombin and adenosine diphosphate plus epinephrine.[36]

In platelet–blood vessel interaction, von Willebrand factor also appears to play an important role. Platelets in whole blood from patients with von Willebrand's disease show decreased adhesion to the vascular subendothelium in a perfusion chamber,[37] and this abnormality can be corrected by purified von Willebrand factor.[38]

If endothelial cells are damaged, many substances in the subendothelial tissues are exposed, including collagens, laminin, fibronectin, vitronectin, elastin, proteoglycans, and microfibrils.

The mechanism by which von Willebrand factor binds to subendothelial tissues is less than clear. Some of the work is conflicting and will not be reviewed here.

Collagen types IV and V seem to be synthesized by endothelial cells. These two types of collagen exist in the subendothelium but do not react with platelets.[39] Type I and III collagen are in the deep layer that may be exposed after severer trauma, and these types of collagen adsorb von Willebrand factor, particularly the large multimers that appear to be associated with shortening of the bleeding time.[40-42] The binding of the platelet to subendothelial tissues has been the subject of much investigation in many laboratories, and evidence is emerging that von Willebrand factor plays a central role in this interaction.[43] Microfibrils can induce platelet aggregation dependent on the level of von Willebrand factor.[44] Microfibrils also prompted the binding of platelets to collagenase-treated subendothelium, and this reaction was inhibited by the monoclonal antibody to platelet GP Ib.[45]

In the present study, the development of the hemostatic plug was investigated, and the volume of the hemostatic plug was calculated using the bleeding time incision in the pig's ear. Our findings in the normal pig can be summarized as follows: Ten seconds after the bleeding time cut had been made, small platelet plugs and platelet aggregates were already present, mainly at the ends of the transected vessels. This finding seems to be the same as reported by Wester et al,[4] in their human study after the template bleeding time test. Immunoperoxidase staining showed a layer of platelets along the surface of the wound channel. This phenomenon has also been reported in the in vitro bleeding time system using normal pig blood and pig skin.[46]

Thirty seconds after the cut had been made, many hemostatic plugs were formed. The volume of the individual plug also increased. About half of these hemostatic plugs were formed at the ends of the transected vessels, most of which were not completely occluded at this stage. Between 30 and 60 seconds, the size and the number of the hemostatic plugs increased dramatically, and fibrin strands began to appear.

These findings suggest the following. Immediately after bleeding starts, circulating platelets begin to adhere to the surface of the cut channel, resulting in formation of a layer of platelets. Then platelet aggregation starts mainly in the vicinity of the transected vessels. Although there were many free platelet clumps in the cut channel, it was not certain whether they were formed at the surface of the incision or whether they were displaced into the wound from the ends of the transected vessels. These aggregated platelets formed larger aggregates with time. Finally, all transected vessels were covered with hemostatic plugs and bleeding stopped.

At 120 seconds, the incisions in two of the five experiments were still bleeding slightly, but there was no difference in number and size of the hemostatic plugs between these two and the other three experiments. Despite the macroscopic arrest of the bleeding, therefore, minor bleeding continued into the incision.

In complete contrast to the normal, large hemostatic plugs were found at seven and 15 minutes in the bleeding time incisions in the von Willebrand pig. The volume of the hemostatic plugs seemed to depend on the duration of the time the tissue was exposed to the blood. However, these large hemostatic plugs did not cover the ends of the transected vessels, so that these vessels continued to bleed, allowing large platelet aggregates to form in the bleeding time incisions. These platelet aggregates were not effective in producing hemostasis, similar to the findings in humans of Hovig and Stormorken.[12] Furthermore, in the large hemostatic plugs, canals that were packed with red blood cells and leukocytes were observed, so it is likely that bleeding continued from the open vessels through these canals.

In our study, cryoprecipitate infusion into a von Willebrand pig corrected the defect of von Willebrand's disease. The bleeding time was shortened from more than 15 minutes to six to eight minutes, and the morphological findings of the hemostatic plugs were similar to those of normal pigs, showing the transected vessels completely covered with the hemostatic plugs. This evidence suggests that the transfused von Willebrand factor could recognize some components in the vessel wall and could mediate the binding of the platelets to the injured vessels.

In a normal pig infused with a monoclonal antibody specific for von Willebrand factor, the ends of the transected small arteries were not covered with the hemostatic plugs at seven minutes, although large hemostatic plugs were found along the surface of the cut channel. At 15 minutes, when bleeding had already stopped, the bleeding time incision was packed by huge hemostatic plugs in which canals were seen. This structure is similar to that found in a pig with von Willebrand's disease.

Localization of the infused monoclonal antibody in the skin of the pig ear was investigated by the PAP staining method. Monoclonal antibody was distributed to the hemostatic plugs, individual platelets adhering to the surface of the cut channel and fibrous tissue, and endothelial cells of the vessels. Among them, hemostatic plugs showed the strongest reaction. Although the monoclonal antibody bound to the plasmatic von Willebrand factor, this immunohistochemical study suggested that it also bound to the local von Willebrand factor in platelets and endothelial cells. Localization of von Willebrand factor in the subendothelium that might be synthesized in endothelial cells[47] has been confirmed by immunofluorescent and immunoelectron microscopic studies, and a role for this local von Willebrand factor in platelet–subendothelium interaction has been suggested.[48]

Our monoclonal W1-8 antibody inhibited adhesion of platelets to the ends of the transected vessels and might bind to the epitope on von Willebrand factor that is responsible for the binding of von Willebrand factor to certain elements of blood vessels or to platelets.

These in vivo findings suggest first that von Willebrand factor is important in the interaction of the platelet and the blood vessel and is involved in the localization of the hemostatic plug to the damaged vessel and its eventual occlusion. Furthermore, large platelet aggregates form in the absence of von Willebrand factor, but these aggregates are friable and penetrated by channels through which bleeding continues. These latter observations suggest that the von Willebrand factor also plays a role in platelet–platelet interaction.

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REFERENCES

1. Sixma JJ, Wester J: The hemostatic plug. Semin Hematol 14:265, 1977
2. Jorgensen L, Borchgrevink CF: The haemostatic mechanism in patients with haemorrhagic diseases. A histological study of wounds made for primary and secondary bleeding time tests. Acta Pathol Microbiol Scand 60:55, 1964
3. Hovig T, Rowsell HC, Dodds WJ, Jorgensen L, Mustard JF: Experimental hemostasis in normal dogs and dogs with congenital disorders of blood coagulation. Blood 30:636, 1967
4. Wester J, Sixma JJ, Geuze JJ, van der Veen J: Morphology of the early hemostasis in human skin wounds. Influence of acetylsalicylic acid. Lab Invest 39:298, 1978
5. Wester J, Sixma JJ, Geuze JJ, Heijnen HFG: Morphology of the hemostatic plug in human skin wounds. Transformation of the plug. Lab Invest 41:182, 1979
6. Kjaerheim A, Hovig T: The ultrastructure of haemostatic blood platelet plugs in rabbit mesenterium. Thromb Diath Haemost 7:1, 1962
7. Borchgrevink CF: A method for measuring platelet adhesiveness in vivo. Acta Med Scand 168:157, 1960
8. Johnson GJ, Schwartz BS, Leis LA: In vivo platelet retention in human bleeding-time wounds. I. Normal subjects and patients with platelet dysfunction. J Lab Clin Med 94:563, 1979
9. Kelton JG, Carter CJ: The effect of aspirin on the size of the hemostatic plug. Thromb Res 32:267, 1983
10. Bloom AL: The von Willebrand syndrome. Semin Hematol 17:215, 1980
11. Zimmerman TS, Ruggeri ZM: Von Willebrand's disease. Clin Haematol 12:175, 1983
12. Hovig T, Stormorken H: Ultrastructural studies on the platelet plug formation in bleeding time wounds from normal individuals and patients with von Willebrand's disease. Acta Pathol Microbiol Scand [Suppl] 248:105, 1974
13. Fass DN, Brockway WJ, Owen CA Jr, Bowie EJW: Factor VIII (Willebrand) antigen and ristocetin-Willebrand factor in pigs with von Willebrand's disease. Thromb Res 8:319, 1976
14. Bowie EJW, Fass DN, Owen CA Jr: Hemostatic effect of transfused Willebrand factor in porcine von Willebrand's disease. Similarities to the human disease. Haemostasis 9:352, 1980
15. Bowie EJW, Thompson JH Jr, Didisheim P, Owen CA Jr: Mayo Clinic Laboratory Manual of Hemostasis. Philadelphia, WB Saunders, 1971
16. Bowie EJW, Owen CA Jr, Zollman PE, Thompson JH Jr, Fass DN: Tests of hemostatis in swine: Normal values and values in pigs affected with von Willebrand's disease. Am J Vet Res 34:1405, 1973
17. Olson JD, Brockway WJ, Fass DN, Bowie EJW, Mann KG: Purification of porcine and human ristocetin-Willebrand factor. J Lab Clin Med 89:1278, 1977
18. Laurell CB: Quantitative estimation of proteins by electrophoresis in agarose containing antibodies. Anal Biochem 15:45, 1966
19. Olson JD, Brockway WJ, Fass DN, Magnuson MA, Bowie EJW: Evaluation of ristocetin-Willebrand factor assay and ristocetin-induced platelet aggregation. Am J Clin Pathol 63:210, 1975
20. Weiss HJ, Hoyer LW, Rickles FR, Varma A, Rogers J: Quantitative assay of a plasma factor deficient in von Willebrand's disease that is necessary for platelet aggregation. Relationship to factor VIII procoagulant activity and antigen content. J Clin Invest 52:2708, 1973
21. Bowie EJW, Owen CA Jr: von Willebrand's disease, in Schmidt RM (ed): CRC Handbook Series in Clinical Laboratory Science, vol 1, sec I, Hematology. Boca Raton, Fla, CRC Press, 1979, p 471
22. Manucci PM, Pareti FI, Holmberg L, Nilsson IM, Ruggeri ZM: Studies on the prolonged bleeding time in von Willebrand's disease. J Lab Clin Med 88:662, 1976
23. Fass DN, Bowie EJW, Owen CA Jr, Zollman PE: Inheritance of porcine von Willebrand's disease: Study of a kindred of over 700 pigs. Blood 53:712, 1979
24. Griggs TR, Webster WP, Cooper HA, Wagner RH, Brinkhous KM: von Willebrand factor: Gene dosage relationships and transfusion response in bleeder swine—A new bioassay. Proc Natl Acad Sci USA 71:2087, 1974

25. Schlicter SJ, Counts RB, Henderson R, Harker LA: Preparation of cryoprecipated factor VIII concentrates. Transfusion 16:616, 1976

26. Mertz ET: The anomaly of a normal Duke's and a very prolonged saline bleeding time in swine suffering from an inherited bleeding disease. Am J Physiol 136:360, 1942

27. Doettl K, Ripke O: Blutgerinnung und Blutungszeit, in Medizin und Chemie, Leverkusen. Germany, Bayer, 1938, p 267

28. Katzmann JA, Mujwid DK, Miller RS, Fass DN: Monoclonal antibodies to von Willebrand's factor: Reactivity with procine and human antigens. Blood 58:530, 1981

29. Sternberger LA: Immunohistochemistry (ed 2). New York, Wiley, 1979

30. Bowie EJW, Fass DN, Katzmann JA: Functional studies of Willebrand factor using monoclonal antibodies. Blood, 62:146, 1983

31. Turitto VT, Weiss HJ, Baumgartner HR: Platelet interaction with rabbit subendothelium in von Willebrand's disease altered thrombus formation distinct from platelet adhesion. JCI 74:1730, 1984

32. Howard MA, Firkin BG: Ristocetin—A new tool in the investigation of platelet aggregation. Thromb Diath Haemorrh 26:363, 1971

33. Coller BS, Peerschke EI, Scudder LE, Sullivan CA: Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: Additional evidence in support of GP Ib as a platelet receptor for von Willebrand factor. Blood 61:99, 1983

34. Ruan C, Tobelem G, McMichael AJ, Drouet L, Legrand Y, Degos L, Kieffer N, Lee H, Caen JP: Monoclonal antibody to human platelet glycoprotein I. II. Effects on human platelet function. Br J Haematol 49:511, 1981

35. Stel HV, Sakariassen KS, Scholte BJ, Veerman ECIL, van der Kwast ThH, deGroot PhG, Sixma JJ, van Mourik JA: Characterization of 25 monoclonal antibodies to factor VIII-von Willebrand factor: Relationship between ristocetin-induced platelet aggregation and platelet adherence to subendothelium. Blood 63:1408, 1984

36. Ruggeri ZM, Marco LD, Gatti L, Bader R, Montgomery RR: Platelets have more than one binding site for von Willebrand factor. J Clin Invest 72:1, 1984

37. Tschopp TB, Weiss HJ, Baumgartner HR: Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J Lab Clin Med 83:296, 1974

38. Weiss HJ, Baumgartner HR, Tschopp TB, Turitto VT, Cohen D: Correction by factor VIII of the impaired platelet adhesion to subendothelium in von Willebrand disease. Blood 51:267, 1978

39. Madri JA, Dreyer B, Pitlick FA, Furthmayer H: The collagenous components of the subendothelium. Correlation of structure and function. Lab Invest 43:303, 1980

40. Santoro SA: Adsorption of von Willebrand factor/factor VIII by the genetically distinct interstitial collagens. Thromb Res 21:689, 1981

41. Santoro SA: Preferential binding of high molecular weight forms of von Willebrand factor to fibrillar collagen. Biochim Biophys Acta 756:123, 1983

42. Kessler CM, Floyd CM, Rick ME, Krizek DM, Lee SL, Gralnick HR: Collagen-factor VIII/von Willebrand protein interaction. Blood 63:1291, 1984

43. Sakariassen KS, Bolhuis PA, Sixma JJ: Human blood platelet adhesion to artery subendothelium is mediated by factor VIII/von Willebrand factor bound to the subendothelium. Nature 279:630, 1979

44. Legrand YT, Fauvel F, Gutman N, Muh JP, Tobelem G, Souchon H, Karniguian A, Caen JP: Microfibrils (MF) platelet interaction: Requirement of von Willebrand factor. Thromb Res 19:737, 1980

45. Fauvel F, Grant MF, Legrand YJ, Souchon H, Tobelem G, Jackson DS, Caen JP: Interaction of blood platelets with a microfibrillar extract from adult bovine aorta: Requirement for von Willebrand factor. Proc Natl Acad Sci USA 80:551, 1983

46. Kimura A, Bowie EJW, Campbell RJ, Fass DN: Willebrand factor in hemostasis in the in vitro bleeding time. Blood 54:1347, 1979

47. Sussman II, Rand JH: Subendothelial deposition of von Willebrand's factor requires the presence of endothelial cells. J Lab Clin Med 100:526, 1982

48. Rand JH, Gordon RE, Sussman II, Chu SV, Solomon V: Electron microscopic localization of factor-VIII-related antigen in adult human blood vessels. Blood 60:627, 1982

The Proportion of Mitoses in Different Cell Lineages Changes During Short-term Culture of Normal Human Bone Marrow

By Mauri Keinänen, Sakari Knuutila, Clara D. Bloomfield, Erkki Elonen, and Albert de la Chapelle

To determine the hematopoietic cell lineage of mitotic cells in human bone marrow on direct examination and after 24-hour culture, marrow mitoses from four healthy individuals were studied, using a new technique that allows analysis of karyotypes in cells whose cell membrane and cytoplasm have been preserved. Mitoses were identified as being of erythroid lineage by immunofluorescent staining for surface glycophorin A and as being of granulocytic lineage by cytoplasmic staining for Sudan black B. On direct marrow examination without prior culture, the great majority of mitoses (74% to 90%) were of erythroid lineage; only a few (0% to 10%) were granulocytic. After 24-hour culture, the percentage of erythroid mitoses (15% to 40%) decreased, while the percentage of granulocytic mitoses (58% to 87%) increased strikingly. These data indicate that mitotic cells of different hematopoietic cell lineages predominate in marrow at different culture times and offer a plausible explanation for the high frequency of normal karyotypes in acute myeloid leukemia after direct marrow cytogenetic evaluation.

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ROUTINE BANDED CHROMOSOME ANALYSIS of marrow does not allow identification of the hematopoietic lineage of the cell from which the metaphase originates. As a result, a number of cytogenetic observations remain unexplained. In acute myeloid leukemia (AML), nonrandom chromosome abnormalities have been found in the bone marrow in anywhere from 40% to nearly 100% of patients.[1-4] Abnormal karyotypes appear to be found more commonly after short-term culture or methotrexate synchronization.[4-7] Even in the presence of cytogenetically abnormal clones, one usually detects cytogenetically normal metaphases. It has been suggested, although not proven, that these findings occur because cells of different lineage are more commonly in mitosis after direct preparation as compared with short-term culture.[8]

A new cytogenetic technique has recently been developed that allows identification of the cell lineage of mitotic marrow cells.[9-10] As a first step to understanding the origin of normal karyotypes in patients with leukemia, we have applied this new technique to normal marrow. We herein show that in healthy subjects, the majority of marrow cells in mitosis after direct examination are erythroid precursors, while after 24-hour culture the majority of mitotic cells are of the granulocyte lineage.

MATERIALS AND METHODS

Subjects. Bone marrow used for this study was obtained from four healthy individuals, aged 22, 25, 27, and 31 years, at the time they donated marrow for transplantation. Informed consent was obtained in accord with University of Helsinki guidelines. Analysis of G-banded chromosomes from both blood and marrow showed only normal karyotypes. Complete medical evaluation demonstrated that the subjects were well within normal hematologic parameters.

Cell cultures and preparation of slides. Our method of preparing mitoses in morphologically and immunologically classified cells has been described in detail previously.[9,10] Samples were obtained directly from the operating room, where aspirated marrow (1 to 2 mL) was immediately placed in RPMI medium that contained 20% fetal calf serum (FCS), L-glutamine (0.29 mg/mL), penicillin (50 U/mL), streptomycin (50 μg/mL), and heparin (4 IU/mL). After 15 minutes, mononuclear cells were isolated using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The mononuclear cells from each subject were divided into two 50-mL Falcon flasks, one for "direct" examination and the other for 24-hour culture. RPMI medium was added to obtain approximately 1 million cells per milliliter. The RPMI medium used was as described earlier but did not contain heparin. In subject 4, the cells were divided into three parts: one for direct examination, one for 24-hour culture, and one for methotrexate synchronization culture.[6] In the methotrexate culture, the cells were incubated for four hours before adding methotrexate; they were then cultured for 17 hours with methotrexate (0.5 μg/mL), washed with thymidine (24 μg/mL), and finally incubated with thymidine for 6.5 hours.

All cultures were incubated at 37 °C in 5% CO_2. In the "direct" examination, the culture time was two hours, throughout which Colcemid (GIBCO, Chagrin Falls, Ohio; 0.125 μg/mL) was present. In the 24-hour culture and the methotrexate culture, Colcemid was present for the two last hours.

Hypotonic treatment with a solution consisting of 50 mmol/L glycerol, 5 mmol/L KCl, 10 mmol/L NaCl, 0.8 mmol/L MgCl, 1 mmol/L $CaCl_2$, and 10 mmol/L sucrose (pH 7.0) followed Colcemid treatment. One milliliter hypotonic solution was mixed with 1 mL cell suspension, and after five minutes at 20 °C, the cells were spun onto slides with a cytocentrifuge. (Shandan Elliot, Runcom, England)

Identification of cell lineages. Cells of granulocytic lineage were identified with Sudan black B (SBB) (Merck) staining. Before staining, the slides were dried at room temperature for one to three days. The cells were fixed with 35% formaldehyde for one minute and washed under running water for two to three minutes. After fixation, the cells were stained with SBB[11] for 30 minutes at room temperature, washed with 70% ethanol for two minutes and with running water for two minutes. To identify the chromosomes, the cells were stained with either Giemsa (Merck, Darmstadt, FRG) or quinacrine as described by Caspersson et al.[12]

An indirect immunofluorescent technique with a monoclonal antibody for glycophorin A (GPA) was used to identify cells of erythroid lineage. The antibody was made against glycophorin isolated from membranes of red cells and kindly provided by Dr L.C. Andersson, University of Helsinki.[13,14] The cells were first treated with methanol-formaldehyde (49.8 mL:0.2 mL) fixation for five

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minutes and then washed with RPMI medium containing 10% FCS. The cells were incubated with antibody at a dilution of 1:10 in a moist, dark chamber for 30 minutes and washed as described. Tetramethylrhodamine (TRITC)-conjugated goat antimouse antiserum (Cappel, Cochranville, Pa) was applied to the cells and they were incubated for 30 minutes. After washing with RPMI containing 10% FCS, the cells were stained with quinacrine to identify the mitotic cells. The specificity of the antibody for GPA was confirmed, using the cell line K562. Negative control slides were prepared as for other immunofluorescent slides, but RPMI medium was used instead of the antibody. Mature erythrocytes served as positive controls. After each staining, positive and negative mitoses for SBB and GPA were counted under the microscope. The total number of mitoses studied was 2,375.

Double staining for SBB and GPA was done in subjects 2 and 3. Cells were stained successively with SBB, anti-GPA, and quinacrine as described. Slides were screened for mitoses using fluorescent light in a fluorescence microscope model Ortholux II (Leitz, Midland, Ontario) with an E2 filter to identify quinacrine-stained chromosomes, which display green fluorescence. To determine how a given mitotic cell reacted with anti-GPA, the filter was changed from E2 to N2. The presence or absence of red fluorescence from TRITC identified the mitosis as, respectively, positive or negative for GPA. Finally, visible light was turned on and the same mitotic cell was scored for SBB staining.

To document the findings, the quinacrine-stained and TRITC-positive cells were photographed on Fujichrome (400 ASA) film with filters E2 for quinacrine and N2 for TRITC. Quinacrine and SBB staining could be visualized and photographed on the same photomicrograph, using simultaneously visible and fluorescent light. The cells stained with Giemsa and SBB were photographed with a Leitz light microscope model Dialux 20 using Ektachrome (50 ASA) film. Black and white figures were made from color slides.

RESULTS

Direct examination. When bone marrow was studied using the direct method, the great majority of mitoses in each subject (74% to 90%) were positive for GPA (Table 1). Only 20 of 576 mitoses studied with SBB from the four subjects were SBB positive. Double staining for SBB and GPA in subject 2 demonstrated that 26% of the mitoses stained neither for SBB nor GPA (Table 2). Double staining of 227 mitoses for both SBB and GPA demonstrated that cells were positive for one or the other but never for both (Fig 1).

Twenty-four-hour culture. After 24-hour culture, the majority of mitoses (58% to 87%) in each subject were SBB positive (Table 1). Only 15% to 40% of mitoses were GPA positive. Similar results were obtained in the one subject (No. 4) studied with the methotrexate synchronization culture method. Here 96.6% of 148 mitoses studied for SBB were positive, whereas no mitoses studied for GPA were positive. Thus, in all four subjects, the lineage of most mitoses shifted from erythroid to granulocytic after 24 hours of culture.

Table 1. Lineage of Mitoses in Normal Bone Marrow After Direct Preparation and 24-Hour Culture

Subject No.	Sex/Age (yr)	SBB- or GPA-Positive Mitoses (%)			
		BM Direct		BM 24-Hour Culture	
		SBB	GPA	SBB	GPA
1	F/27	0.0 (51)	87.5 (64)	87.3 (63)	20.0 (15)
2	M/22	1.1 (180)	73.6 (91)	80.8 (203)	14.8 (176)
3	M/25	2.3 (218)	81.8 (110)	57.5 (360)	40.2 (328)
4	F/31	10.2 (127)	90.4 (52)	87.2 (133)	18.8 (80)
Total		3.5 (576)	82.0 (317)	71.4 (759)	29.4 (599)

The number of mitoses studied is in parentheses. SBB, Sudan black B; GPA, glycophorin A; BM, bone marrow.

Table 2. Double Staining of Single Mitotic Cells for Sudan Black B and for Glycophorin A

Subject No.	Mitoses With Designated Reactivity (%)					
	BM Direct			BM 24-Hour Culture		
		SBB +	SBB −		SBB +	SBB −
2	GPA +	0	74 (55)*	GPA +	0	12 (17)
	GPA −	0	26 (19)	GPA −	84 (124)	4 (6)
3	Not done			GPA +	0	34 (44)
				GPA −	58 (75)	9 (11)

*The number of mitoses with the stated reactivity is in parentheses (eg, 55 mitoses were negative for SBB and positive for GPA).

Double staining in subjects 2 and 3 showed 4% and 9%, respectively, of mitoses to be negative for both SBB and GPA (Table 2). In all four subjects, the percentage of SBB plus GPA positive cells increased. In the one subject (No. 2) studied with double staining in both direct and 24-hour culture, the percentage of unidentified cells decreased (Table 2).

DISCUSSION

We have shown an increase in SBB-positive mitoses and a decrease in GPA-positive ones after 24-hour culture. SBB is a histologic lipid stain that blackens granules of both mature and immature granulocytes.[15,16] SBB positivity can usually be seen in myeloblasts, even when they have a small number of granules. During maturation through the myelocyte and metamyelocyte stages of development, the staining power is augmented. Eosinophil granules are likewise sudanophilic. Monocytes may be unstained or many contain a few discrete sudanophilic granules. Basophil granules are found to be sudanophobic in reaction, as are also azur granules of lymphoid cells, both remaining unstained. Cells of megakaryocytic and erythroid lineage also remain unstained. SBB staining is thus usable to relatively selectively identify almost all mitoses of granulocytic lineage, even though it may miss early granulocytic precursors because they do not yet contain enough lipid granules to stain with SBB.

GPA is the major membrane sialoglycoprotein on mature erythrocytes.[17] This protein is found only at the cell surface on cells of the erythroid lineage in normal bone marrow.[13,14] GPA occurs in basophilic normoblasts and later stages of erythrocyte differentiation, whereas pronormoblasts do not seem to contain it. The surface expression of GPA occurs slightly earlier than the onset of hemoglobin synthesis and the appearance of the ABO blood group antigens during normal red cell differentiation. GPA is a selective marker for cells of erythroid lineage; consequently, cells of erythroid origin can be identified in bone marrow with GPA antibodies.

We believe that our evaluation of metaphase cells for SBB and GPA clearly demonstrates that when direct chromosome preparations are studied from normal marrow, one is primarily studying chromosomes from cells of erythroid lineage. Ten percent or less of chromosome metaphases would appear

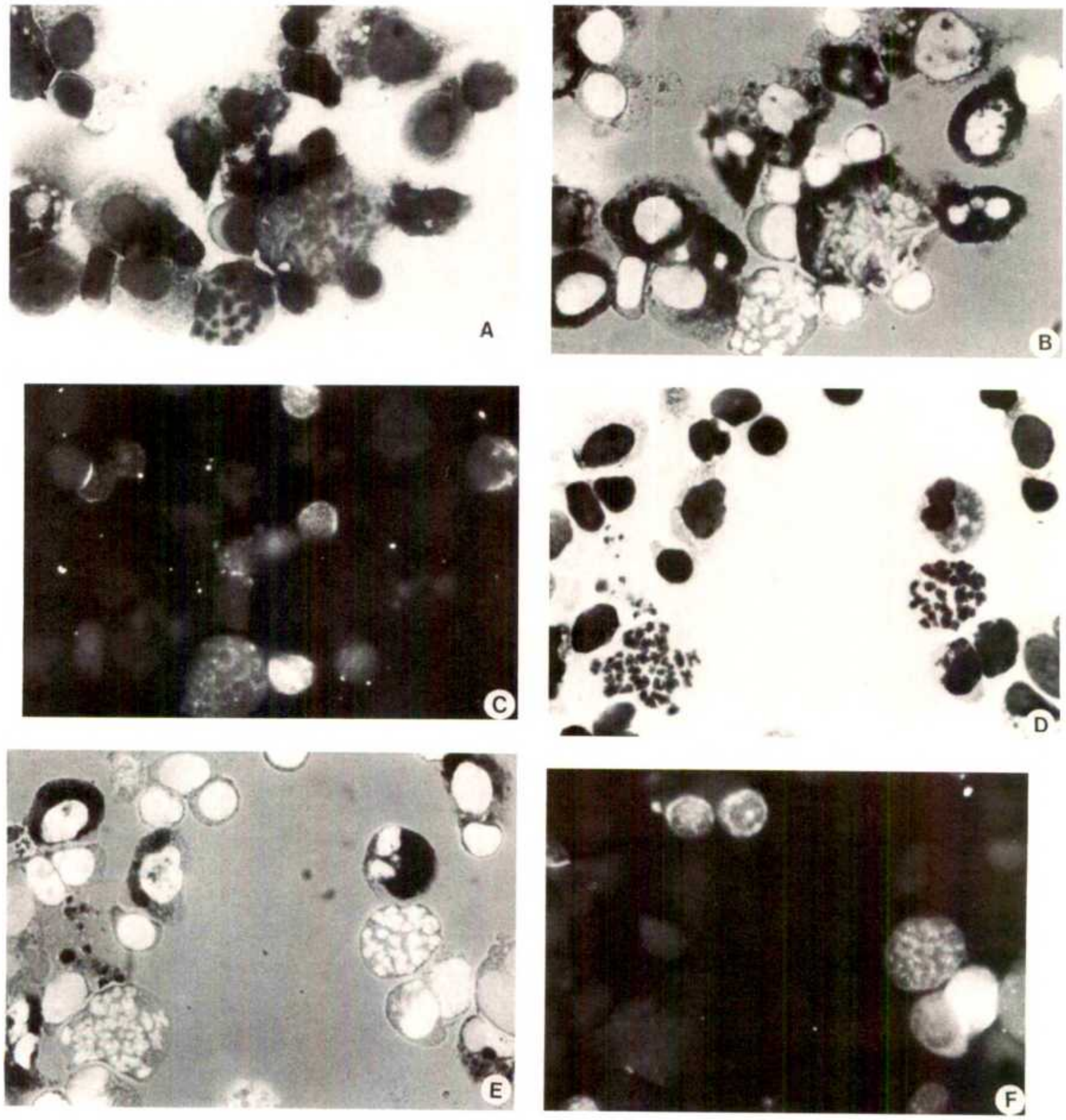

Fig 1. Twenty-four-hour cultured bone marrow cells from subject 2. Two microscopic fields (A through C; D through F) each containing two cells in mitosis are shown. Cells were stained with Giemsa (A, D), Sudan black B plus quinacrine (B, E), and for GPA (C, F). Giemsa staining was done after the other stainings, and thus chromosome morphology is not optimal. In field 1 (A through C), the mitosis on the right is SBB positive (B) and GPA negative (C) and the mitosis on the left, GPA positive (C) and SBB negative (B). In field 2 (D through F), the mitosis on the right is GPA positive (F) and SBB negative (E); the mitosis on the left is negative for both. Original magnification ×800; current magnification ×520.

to be of granulocytic lineage. These results are consistent with early studies of routine marrow aspirates, which found 65% to 75% of mitotic cells to be erythroid.[18-20] Most of the remaining mitotic cells were found to be of the neutrophil series.[18,20] In one study, the myeloid-erythroid ratio was as low as 48:52.[21] These somewhat higher frequencies of granulocytic precursors found in the early studies may relate to the differences between cells in mitosis after procedures for preparation of metaphase chromosomes, eg, two-hour incubation in the presence of Colcemid as compared with morphologically evaluating aspirated marrow immediately smeared onto a slide.

Our data show that karyotypes evaluated after 24-hour culture come mostly from the granulocytic series, although karyotypes from erythroid precursors are still present. Because monocytes and eosinophils and their precursors also stain with SBB, a small proportion of the karyotypes probably comes from cells of these lineages. We know of no other studies of the lineage of mitotic cells in normal marrow after 24-hour culture. In one study, the frequency of polyploid mitoses (and thus presumably cells of megakaryocytic lineage) in healthy subjects was found to increase after incubation of bone marrow cells for six hours[22]; bone marrow after 24 hours of culture was not studied. We seldom saw polyploid mitoses after either direct or 24-hour culture.

Our data suggest that on direct marrow examination, as many as one quarter of the chromosome metaphases studied may be from cells of other than the erythroid or granulocytic lineage. Double staining of direct marrow in subject 2 found 26% of the mitoses to be negative for both SBB and GPA; experiments on the remaining three subjects, using separate slides for the scoring of mitotic cells for GPA and SBB, suggested that up to 20% of cells might be of other than erythroid or granulocytic lineage. After culture, more than 90% of chromosome metaphases appear to come from cells of erythroid or granulocytic lineage. To date, we have not been able to determine the lineage of these GPA-, SBB-negative mitotic cells. These could obviously be of lymphoid, monocytic, or megakaryocytic lineage or be very early granulocytic precursors that do not stain for SBB or very early erythroid precursors that do not show GPA.

The results of our study directly show that for normal bone marrow, the erythroid mitoses decrease, while the mitoses of granulocytic origin increase after short-term culture. This may explain why in some cases of leukemia no chromosome abnormalities are found on direct study, but appear after short-term culture. By the use of different culture times, proportions of normal and abnormal mitoses may vary greatly. Extrapolating our results to leukemia indicates the possibility that at different times of culture, one may be studying different cell lines whose time of peak mitotic activity varies. Indeed, Berger et al[8,23] have data in AML suggesting that this is the case. Investigating in parallel bone marrow specimens from patients with AML cytologically and cytogenetically, they observed that in direct bone marrow preparations, there were many erythrocytes in mitosis, but after culture, these decreased in number. Simultaneously, the proportion of cells with a normal karyotype diminished after culture.

Although we have studied only one individual with the methotrexate synchronization culture method, the data are of interest because in this case, essentially all of the mitotic cells were of granulocytic lineage and none were of erythroid lineage. If the data are confirmed by study of further individuals, this might explain the higher frequency of identification of the clonal chromosome abnormality found by some in AML after methotrexate[4,24] and fluorodeoxyuridine[25] synchronization.

REFERENCES

1. Berger R, Bloomfield CD, Sutherland GR: Report of the committee on chromosome rearrangements in neoplasia and on fragile sites. Eighth International Workshop on Human Gene Mapping. Cytogenet Cell Genet 40:452, 1985

2. Rowley JD: Biological implications of consistent chromosome rearrangements in leukemia and lymphoma. Cancer Res 44:3159, 1984

3. Golomb HM: Chromosome abnormalities in adult acute leukemia: Biologic and therapeutic significance, in Bloomfield CD (ed): Adult Leukemias, Vol 1. Boston, Martinus Nijhoff, 1982, p 127

4. Yunis JJ, Bloomfield CD, Ensrud K: All patients with acute nonlymphocytic leukemia may have a chromosomal defect. N Engl J Med 305:135, 1981

5. Knuutila S, Vuopio P, Borgström GH, de la Chapelle A: Higher frequency of 5q- clone in bone marrow mitoses after culture than by direct method. Scand J Haematol 25:358, 1980

6. Knuutila S, Vuopio P, Elonen E, Siimes M, Kovanen R, Borgström GH, de la Chapelle A: Culture of bone marrow reveals more cells with chromosomal abnormalities than the direct method in patients with hematologic disorders. Blood 58:369, 1981

7. Waghray M, Eques G, Rowley JD, Martin P, Testa JR: Methods of processing marrow samples may affect the frequency of detectable aneuploid cells. Am J Hematol 11:409, 1981

8. Berger R, Bernheim A, Daniel MT, Valensi F, Flandrin G: Cytological types of mitoses and chromosome abnormalities in acute leukemia. Leuk Res 7:21, 1983

9. Teerenhovi L, Knuutila S, Ekblom M, Rossi L, Borgström GH, Tallman JK, Andersson L, de la Chapelle A: A method for simultaneous study of the karyotype, morphology and immunologic phenotype of mitotic cells in hematologic malignancies. Blood 64:1116, 1984

10. Knuutila S, Keinänen M: Chromosome banding techniques for morphologically classified cells. Cytogenet Cell Genet 39:70, 1985

11. Undritz E: Staining of blood and bone marrow films. Atlas of Haematology. Sandoz, Basel, Switzerland, Frobenius Ltd, 1973, p 38

12. Caspersson T, Zech L, Johansson C, Modest EJ: Identification of human chromosomes by DNA-binding fluorescing agents. Chromosoma 30:215, 1970

13. Gahmberg CG, Jokinen M, Andersson LC: Expression of the major sialoglycoprotein (glycophorin) on erythroid cells in human bone marrow. Blood 52:379, 1978

14. Andersson LC, von Willebrand E, Gahmberg CG, Siimes M, Vuopio P: Glycophorin A and fetal hemoglobin as markers of early erythroid derivation in human leukemias, in Knapp W (ed): Leukemia Markers. Proceedings of the Leukemia Marker Conference. London, Academic Press, 1981, p 249

15. Sheehan HL, Storey GW: An improved method of staining leucocyte granules with Sudan-black B. J Pathol Bacteriol 59:336, 1947

16. Nelson DA, Davey FR: Sudan black B staining, in Williams WJ, Beutler E, Erslev AJ, Rundles RW (eds): Hematology. New York, McGraw-Hill, 1977, p 1629

17. Marchesi VT, Tillack TW, Jackson RL, Segrest JP, Scott RE: Chemical characterization and surface orientation of the major glycoprotein of the human erythrocyte membrane. Proc Natl Acad Sci USA 69:1445, 1972

18. Videbaek A: Normal bone marrow punctuates from individuals in various age groups. Folia Haematol 65:203, 1941

19. Dacie JV, White JC: Erythropoiesis with particular reference to its study by biopsy of human bone marrow: A review. J Clin Pathol 2:1, 1949

20. Killman S-A, Cronkite EP, Fliedner TM, Bond VP: Mitotic indices of human bone marrow cells. I. Number and cytologic distribution of mitoses. Blood 19:743, 1962

21. Lala PK, Maloney MA, Patt HM: A comparison of two markers of cell proliferation in bone marrow. Acta Haematol 31:1, 1964

22. Knuutila S, Simell O, Lipponen P, Saarinen I: Bone-marrow chromosomes in healthy subjects. Hereditas 82:29, 1976

23. Berger R, Bernheim A, Daniel MT, Valensi F, Sigaux F, Flandrin G: Cytologic characterization and significance of normal karyotypes in t(8;21) acute myeloblastic leukemia. Blood 59:171, 1982

24. Testa JR, Misawa S, Oguma N, van Sloten K, Wiernik PH: Chromosomal alterations in acute leukemia patients studied with improved culture methods. Cancer Res 45:430, 1985

25. Levin MD, Garson OM: The value of bone marrow cell synchronization. Cancer Genet Cytogenet 13:93, 1984

Deformability and Intrinsic Material Properties of Neonatal Red Blood Cells

By Otwin Linderkamp, Gerard B. Nash, Paul Y.K. Wu, and Herbert J. Meiselman

Whole cell and membrane deformability are essential for red blood cell (RBC) survival and for effective blood flow. Neonatal RBCs display several specific properties (eg, large size, high hemoglobin F) that could influence their deformation characteristics and contribute to their shortened life span. The present study was designed to compare selected rheologic properties (cellular deformability, pressure required to aspirate RBC into micropipettes, static and dynamic viscoelastic material properties) of neonatal and adult RBCs. RBC deformability, as studied by a rheoscope, was similar for neonates and adults over a shear stress range of 2.5 to 500 dyn/cm^2. The pressure required to aspirate RBCs completely into 3.3-μm diameter pipettes was 129 ± 87 dyn/cm^2 for neonatal RBCs and 71 ± 37 dyn/cm^2 for adult RBCs. The aspiration pressure for neonatal and adult RBCs increased with increasing RBC volume, suggesting that the increased mean aspiration pressure for neonatal RBCs resulted from their larger volume. When RBCs with same volume and diameter were compared, the aspiration pressure tended to be smaller for neonatal RBCs than for adult cells. To characterize material properties determining RBC deformability, we measured membrane extensional (shear) and bending elastic moduli, the time constant for elastic recovery from extensional deformation and hemoglobin viscosity (ie, cytoplasmic viscosity) of neonatal and adult RBCs. Membrane surface viscosity and time constant for recovery from bending deformation were calculated. The extensional and bending moduli of neonatal RBCs were slightly smaller (10% and 16%, respectively) compared with adult cells. This suggests that the static resistance of neonatal RBC membrane to deformation and failure in response to a given force is slightly smaller. The time constant for recovery from extensional deformation of neonatal RBCs was larger by 14%, compared with adult cells. The time constant for bending deformation related to the RBC diameter and surface area was increased by 18% in the neonates. Membrane surface viscosity and hemoglobin viscosity were similar for both cell types. These results indicate that the deformability and viscoelastic properties of neonatal RBCs deviate only slightly from those of adult RBCs and that the increased aspiration pressure of neonatal RBCs is solely due to their large size. Some of the specific deformation characteristics observed in this study (increased aspiration pressure, decreased resistance to elastic deformation) may contribute to the shortened life span of neonatal RBCs.

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RED CELL DEFORMABILITY is essential for effective blood flow, particularly in the microcirculation, where the 8-μm diameter red blood cells (RBCs) must negotiate 3- to 4-μm diameter vessels.[1] RBC deformability also influences blood flow in large vessels with rapid flow, since deformability enables RBCs to adapt to high shear forces, thereby lowering blood viscosity.[2] The capacity of RBCs to survive in the vascular system is a consequence of both the ability of RBCs to deform and to resist overextension and fragmentation of the membrane in shear deformation.[3]

Filtration techniques are widely used as models of the microcirculation and for measurement of RBC deformability. Because neonatal RBCs are less filterable than adult RBCs,[4-6] it has been suggested that neonatal RBCs are less deformable.[4,5] Decreased RBC deformability could contribute to both the markedly shortened life span of neonatal RBCs[7] and to the susceptibility of neonates to serious circulatory problems.[8] On the other hand, neonatal and adult RBCs show no difference in deformability when studied under defined shear forces by means of a cone-plate viscometer,[8] a rheoscope,[9] or an ektacytometer.[10] These apparently contradictory results may be due to the fact that RBC filterability depends on RBC size in addition to RBC deformability, whereas the other methods measure RBC deformability independent of RBC size. Aspiration of RBCs into micropipettes with diameters of about 3 μm provides a relevant model to study both the deformability and volume of individual RBCs as they enter narrow capillaries.[11] Such micropipette studies do not appear to exist for neonatal RBCs.

Cellular deformability is determined by extrinsic properties (overall cellular shape and excess surface area) and by intrinsic material properties (resistance of the membrane to extension and bending forces, and cytoplasmic viscosity).[12-14] The intrinsic properties are of primary importance for the RBC deformation in small-geometry vessels.[14] When an RBC enters a narrow capillary, pore, or micropipette, it is deformed by extension and folding. Three processes are involved in this kind of deformation[14]: (1) membrane extension without change in surface area (resisted by extensional or shear rigidity); (2) membrane bending and folding (resisted by bending rigidity); (3) shear and displacement of the cell contents (resisted by hemoglobin viscosity). When an RBC is aspirated into a narrow micropipette by increasing suction pressure, deformation begins with a smooth membrane extension until, at a specific suction pressure, the membrane buckles and folds and the cell rapidly enters the pipette.[15] Because extensional rigidity (defined by extensional elastic modulus) is more than 10^9 times greater than bending rigidity (defined by bending elastic modulus),[14,15] the pressure at which extension is followed by folding should be important for the pressure required to aspirate the entire cell.

Models have been developed to measure and analyze both

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extensional[16,17] and bending[14,15] deformations. In these models, static rigidities of the RBCs are defined by elastic moduli for membrane extension and bending. Dynamic rigidities are characterized by time constants for elastic recovery from extension and bending deformations. The static rigidities limit the ability of a cell to enter a small channel at given pressures, whereas the dynamic rigidities influence the rate of entry. The time constants are inversely proportional to the corresponding elastic moduli and directly proportional to the membrane surface viscosity or hemoglobin viscosity[14] (see Materials and Methods).

Both extension and bending characteristics can be studied by well-controlled micropipette aspiration techniques,[14-17] but the resulting data are subject to uncertainties if too high pressures are used. For example, Smith et al[18] aspirated RBC membrane tongues into pipettes with diameters of 2 μm using a suction pressure of 20 cm H_2O (20,000 dyn/cm^2) and into 1-μm pipettes using a pressure of 5 cm H_2O (5,000 dyn/cm^2). They observed longer tongue lengths for neonatal RBCs than for adult RBCs and concluded that neonatal RBCs are more deformable and less resistant to shearing than adult cells.[19] However, the pressures that were used were far beyond those required to extend and fold the membrane itself,[15] and thus the tongue length would be more closely related to the absolute excess surface area, which is known to be larger for the neonatal RBC.[20]

The present study was designed to evaluate the extensional and bending moduli and time constants and the membrane and hemoglobin viscosities of neonatal and adult RBCs by means of micropipette and viscometry techniques. Micropipettes with 3.3-μm diameter were used to measure the pressure required to aspirate whole RBCs. A rheoscope was used to determine RBC deformation at defined shear forces.

MATERIALS AND METHODS

Blood samples and RBC preparation. A total of 35 healthy term neonates with gestational ages of 39 to 41 weeks and birth weights of 3,300 to 3,590 g were studied with approval of the University of Southern California Human Subjects Research Committee. Placental blood samples were taken from the umbilical cord immediately after cord clamping and before delivery of the placenta. Adult blood samples were collected from 35 healthy laboratory and hospital personnel by venipuncture. Blood samples from the neonates and the adults were anticoagulated with heparin (5 U/mL blood). All measurements were made at room temperature (22 ± 1 °C) within four hours after collection.

Whole blood was not centrifuged and washed before dilution in the appropriate suspending medium, since neonatal RBCs are sensitive to morphological transformation and mechanical damage.[10,21] A part of each blood sample was used for analyses of hematologic indices. For the micropipette studies, a portion of the blood was diluted 1:500 (hematocrit about 0.1%) in phosphate-buffered saline (PBS; 0.005 mol/L KH_2PO_4 + Na_2HPO_4, 0.153 mol/L NaCl, pH 7.40, 300 mosm/kg) containing 1 g/L of human serum albumin. Another portion of the blood was centrifuged to obtain plasma. The removed plasma was centrifuged a second time at 20,000 g for 15 minutes to remove platelets. For the rheoscope deformability measurements, blood was diluted 1:50 (hematocrit about 1%) in PBS containing 20 g/dL of dextran T-70 (Pharmacia Fine Chemicals, Uppsala, Sweden). The osmolality of these dextran solutions was 295 ± 5 mosm/kg and the viscosity was 21 cP at 25 °C. For the measurement of hemoglobin viscosity, a portion of the blood was washed three times with albumin-free PBS and then processed to obtain membrane-free hemoglobin solutions (see later). All solutions and plasma samples were filtered through 0.45-μm Millipore filters before use.

Micropipette system. The microscope-video and micropipette system were as described elsewhere.[13] A precision micrometer allowed 0.01 mm H_2O (0.98 dyn/cm^2) pressure resolution. The micropipettes were electrically grounded to prevent electrophoretic RBC motion.[22] Micropipettes were initially filled with plasma of the donor and then flushed with the albuminated PBS used to suspend the RBCs. This procedure prevents RBC adhesion to the glass walls of the pipette.[22] One neonatal and one adult blood sample were studied on the same day, using the same micropipette; 30 RBCs were tested in each sample. Resting diameters of all RBCs were determined before their aspiration. Micropipettes with three diameters were used: (1) 3.3-μm pipettes for aspiration pressure and cellular volume; (2) 2-μm pipettes for bending elastic modulus and surface area; (3) 1-μm pipettes for extensional elastic modulus and recovery time, and bending elastic modulus.

Aspiration of whole RBCs. Micropipettes with a tip diameter of 3.3 μm were used to measure the aspiration pressures required to draw RBCs completely into the pipettes.[11] The suction pressure was applied in increments of 0.1 mm H_2O (9.8 dyn/cm^2) and each pressure was held for periods of two to three seconds. Suction pressures were increased to a maximum of 5 mm H_2O. Few cells failed to enter the pipettes at a pressure of 5 mm H_2O (Fig 1); for these cells, the aspiration pressure was assumed to be 5 mm H_2O. These incompletely aspirated cells were then released by decreasing the pressure, and because they moved out freely, adhesion to the glass walls of the pipette appeared to be minimal or nonexistent.

Cellular volume of the completely aspirated cells was calculated from the cell length (L) in the pipette and the pipette radius (R):

$$V = (\pi \cdot R^2 \cdot L) - (2/3\pi R^3). \qquad [1]$$

The volume of incompletely aspirated cells was calculated from the length (L) of the aspirated cell portion, the radius (R_s) of the spherical outer portion, and the pipette radius (R)[13]:

$$V = (\pi/3)\,(3LR^2 + 4R_s^3 - R^3). \qquad [2]$$

The resting diameter (D) of each RBC was measured before aspiration and the mean thickness (δ) calculated from:

$$\delta = (4V)/(\pi\, D^2). \qquad [3]$$

Extensional elastic modulus and recovery time. Time constant for extensional shape recovery (t_e) and the membrane extensional (shear) modulus (μ_e) were measured and analyzed by the methods of Hochmuth et al[17] and Evans and LaCelle[16] using micropipettes with internal diameters of 0.9 to 1.1 μm. The time constant was determined by using the pipette to extend (length-width ratio approximately 1:6) and quickly release point-attached RBCs.[17] Note that extension of the cell by this procedure causes little buckling or curvature change of the membrane surface and is thus mainly the result of elastic shear deformation. From video recordings of the recovery process, the cell length-width ratio was measured at the first data point after releasing the cell, $(L/W)_m$, and then every 1/60 second for 20 fields (L/W). The final value was reached after 0.33 second $(L/W)_0$. The time constant (t_e) was calculated using the equation derived by Hochmuth et al[17]:

$$\frac{(L/W) - (L/W)_0}{(L/W) + (L/W)_0} \cdot \frac{(L/W)_m + (L/W)_0}{(L/W)_m - (L/W)_0} = e^{-t/t_e}, \qquad [4]$$

where t is time in seconds after release of the RBCs.

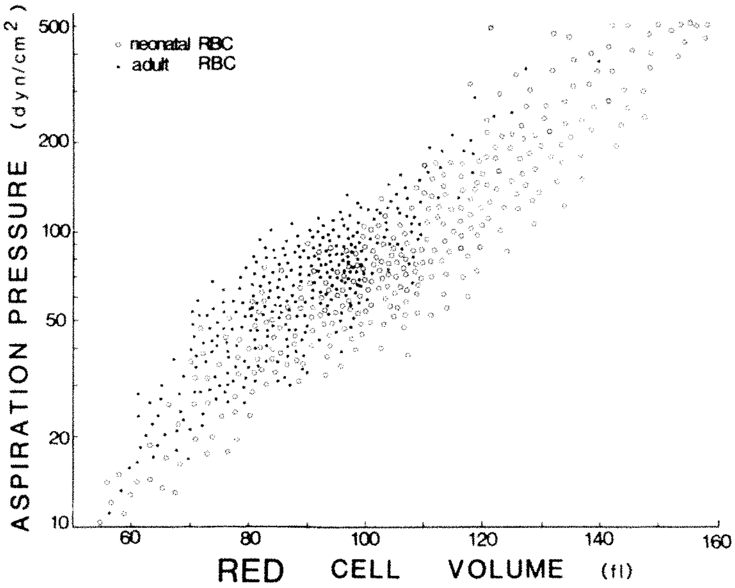

Fig 1. Pressure required to aspirate whole red blood cells (RBCs) into 3.3-μm diameter pipettes plotted against volume of aspirated RBCs. Note that at a given RBC volume, neonatal RBCs tended to need lower aspiration pressures than adult RBCs. Few neonatal cells failed to enter the pipettes at a pressure of 500 dyn/cm².

The extensional (shear) elastic modulus (μ_e) was measured after the extended cell had recovered its initial shape. The same cell was detached from the coverslip, and a small membrane tongue was aspirated into the micropipette from a flat portion of its surface. The pipette pressure was adjusted to zero and then increased in 1-mm H_2O (98 dyn/cm²) steps until the cell began to fold. The length of the membrane tongue (L_t) in the pipette was measured at each pressure (P) from video recordings. A linear relationship exists between L_t/R and $P \cdot R$ (R, pipette radius) over a L_t/R range approximately between 1 and 3. Therefore, a linear approximation[23] to the theory developed by Evans and La Celle[16] for analysis of this system was used:

$$(P \cdot R)/\mu_e = 2.45\,(L_t/R) - 0.603. \qquad [5]$$

The product of the time constant (t_e) for extensional shape recovery and the membrane extensional modulus (μ_e) is determined by viscous dissipation during shape recovery[17]:

$$\mu_e \cdot t_e \approx \eta_m + \eta_{Hb} \cdot \delta, \qquad [6]$$

where η_m and η_{Hb} are the viscosities of the membrane surface (dyn · s/cm) and the cytoplasm (dyn · s/cm²), respectively, and δ is the mean RBC thickness (cm). Because for normal adult RBCs, $\eta_{Hb} \cdot \delta$ is small compared with η_m, and because neonatal and adult RBCs have similar mean thicknesses[20] (Table 1) and hemoglobin viscosities (Table 2), membrane surface viscosities for both cell types were calculated as:

$$\eta_m = t_e \cdot \mu_e. \qquad [7]$$

Bending elastic modulus and recovery time. Membrane bending elastic modulus (ie, resistance to membrane bending and folding) can be derived from measurement of the minimum pressure required to induce membrane buckling in a micropipette.[15] During RBC aspiration into a narrow pipette, the suction pressure produces a uniform membrane extension until the critical "buckling pressure" is reached; beyond this critical point, the cell becomes unstable and folds. Cell instability and folding can easily be seen from the side of the pipette during measurement of the membrane extensional modulus, whereas the beginning of buckling can only be observed by using curved pipettes. However, Evans[15] found that buckling consistently begins at pressures about 60% of the level required to fold the whole cell, and thus the initial buckling pressure can be estimated from the initial folding pressure. The bending elastic modulus (μ_b) is calculated from the initial buckling pressure (P_b) and the radius of the pipette (R):

$$\mu_b = P_b \cdot R^3 \cdot C_b,$$

$$C_b = \frac{m^2 - (n\pi/\ln \tilde{R}_0)^2 - 1}{2[(n\pi/\ln \tilde{R}_0)^4 + 2(n\pi/\ln \tilde{R}_0)^2(m^2 + 1) + (m^2 - 1)^2]}. \qquad [8]$$

The terms $\tilde{R}_0$, and the modal coefficients m and n are defined as:

$$\tilde{R}_0 = R_0/R,$$

where R_0 is the radial distance to the toroidal rim of the red cell:

$$m > (n\pi/\ln \tilde{R}_0) + 1,$$

$$n = 1.$$

Table 1. Hematologic and Whole Red Cell Deformability Data

	n	Neonatal RBCs	Adult RBCs
MCV (fl)	35	107.6 ± 5.8*	89.4 ± 4.9
MCH (pg)	35	35.1 ± 2.5*	29.5 ± 2.0
MCHC (g/dL)	35	32.6 ± 1.7	33.0 ± 1.4
Diameter (μm)	25	8.7 ± 0.4* (± 0.7)*	7.8 ± 0.3 (± 0.6)
Hemoglobin F (%)	10	69.2 ± 8.9*	0.7 ± 0.3
Deformation at 50 dyn/cm² (rheoscope)	20	0.41 ± 0.03 (± 0.06)	0.42 ± 0.03 (± 0.06)
Aspiration pressure (dyn/cm²) for 3.3-μm pipettes	10	129 ± 20 (± 87)	71 ± 9 (± 37)
Volume of aspirated RBCs (fl)	10	111.3 ± 6.9* (± 20.4)†	94.9 ± 5.0 (± 13.7)
Mean RBC thickness (μm)	10	1.83 ± 0.11 (± 0.29)	1.92 ± 0.13 (±0.33)
RBC surface area (μm²)	5	149.4 ± 7.5* (± 21.0)	132.1 ± 6.7 (± 14.1)

Neonatal and adult samples were studied. Data represent mean ± 1 SD. Note that the first SD reflects the spread of the sample means, whereas the values in parentheses reflect the spread of the entire RBC populations studied in each group.

*$P < .005$ when compared with adult RBCs (two-tailed unpaired *t* test, 2*n*-2 degrees of freedom.

†$P < .05$ when compared with adult RBCs (two-tailed unpaired *t* test, 2*n*-2 degrees of freedom).

Table 2. Viscoelastic Properties of Neonatal and Adult RBCs

	n	Neonatal RBC	Adult RBC
Shear (extensional) elastic modulus (10^{-3} dyn/cm)*	10	5.4 ± 0.6‡ (± 1.3)	6.0 ± 0.5 (± 1.1)
Time constant for extensional recovery (s)*	10	0.138 ± 0.017‡ (± 0.026)‡	0.121 ± 0.015 (± 0.023)
Calculated surface viscosity (10^{-3} dyn · s/cm)*	10	0.75 ± 0.14 (± 0.20)	0.73 ± 0.12 (± 0.18)
Bending elastic modulus (10^{-12} dyn/cm)*	10	1.38 ± 0.18‡ (± 0.28)‡	1.64 ± 0.20 (± 0.25)
Bending elastic modulus (10^{-12} dyn/cm)†	5	1.35 ± 0.17‡ (± 0.24)	1.60 ± 0.18 (± 0.23)
Calculated time constant for bending recovery (t_b, *s*)*	10	0.41 ± 0.07‡ (± 0.11)‡	0.31 ± 0.06 (± 0.08)
t_b/diameter (s/cm)*	10	462 ± 78‡ (± 120)	391 ± 62 (±82)
t_b (s)†	5	0.42 ± 0.07‡ (± 0.12)	0.32 ± 0.05 (± 0.09)
t_b/surface area (s/cm^2)†	5	2798 ± 461 (± 805)	2372 ± 346 (± 613)
Hemoglobin viscosity at 33 g/dL (cP)	10	6.6 ± 0.9	6.4 ± 0.8

Neonatal and adult samples were studied. Data represent mean ± 1 SD. Note that the first SD reflects the spread of the sample means, whereas the values in parentheses reflect the spread of the entire RBC populations studied in each group.

*Data from studies using 1-μm diameter pipettes.

†Data from studies using 2-μm diameter pipettes.

‡$P < .05$ when compared with adult RBCs (two-tailed unpaired *t* test, 2*n*-2 degrees of freedom).

C_b was determined for each RBC. It was 0.018 for the average adult RBC and 0.022 for the average neonatal RBC for pipettes with a diameter of 1 μm. In the present study, the bending elastic modulus was estimated from the pressure required to initiate folding of whole RBCs by using micropipettes with internal diameters of approximately 1 and 2 μm (Fig 2); the folding pressures were multiplied by a factor of 0.6 to yield the initial buckling pressures. The 1-μm pipettes were used to determine both the extensional and bending elastic modulus for the same cell. The 2-μm pipettes were used to determine the cellular surface area by increasing the suction pressure to 15 mm H_2O [13,20] (1,500 dyn/cm^2) after the folding pressure was measured.

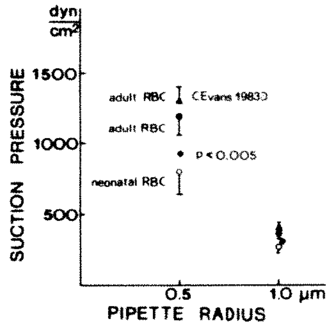

Fig 2. Suction pressure at red blood cell (RBC) folding plotted against pipette inner radius. Data from the present study (open and closed circles) are means ± SD for 300 neonatal and 300 adult RBCs (0.5 μm) and for 150 neonatal and 150 adult RBCs (1.0 μm). Thirty cells from each donor were studied.

The time constant for bending recovery was not measured in the present study. This time constant can, however, be approximated from the hemoglobin viscosity (η_{Hb}), RBC diameter (D), bending elastic modulus (μ_b), and the curvature of the fold (C)[14]:

$$t_b \approx (\eta_{Hb} \cdot D)/(\mu_b \cdot C^2). \qquad [9]$$

The dimensionless term C has a value of about 10^4, and the hemoglobin viscosity of neonatal and adult RBCs is approximately 6.5 cP (Table 2). Thus, t_b could be estimated from D and μ_b for individual RBC:

$$t_b = (6.5 \cdot 10^{-10})\,(D/\mu_b). \qquad [10]$$

Hemoglobin viscosity. Hemoglobin solutions were prepared from RBCs washed and packed according to the Cokelet and Meiselman[24] method. The hemoglobin concentration was adjusted at 33 g/dL with albumin-free PBS and the viscosity was measured by means of a model LVT Wells-Brookfield Cone Plate Microviscometer (Brookfield Eng Lab, Stoughton, Mass) at 22 °C. The instrument was calibrated with silicone oils of known viscosity before the hemoglobin solution measurements.

RBC deformability in the rheoscope. The deformability of a single RBC was observed and measured using a counterrotating, cone-plate rheoscope[2] (Effenberger, Munich), which was mounted on an inverted microscope (Leitz Diavert, Leitz, Wetzlar, Germany); details of the method have been described elsewhere.[9] Ten shear stresses from 2.5 to 500 dyn/cm^2 were applied, and the length (L) and width (W) of 40 RBCs were measured in each sample at each shear stress. The RBC deformation parameter (D) is defined as $D = (L - W)/(L + W)$.

Miscellaneous techniques. Solution osmolalities were determined by freezing-point depression and pH by way of Radiometer systems (Radiometer, Copenhagen) operating at 25 °C. RBC counts and mean cell volumes (MCV and sample SD) in isotonic, filtered PBS were measured electronically with a computerized Electrozone-Celloscope (Particle Data Inc, Elmhurst, Ill) equipped with a 76-μm orifice and operating with a 20% rejecting level to eliminate the artifact caused by nonaxial transit of the RBCs. Hemoglobin concentrations were determined spectrophotometrically by the cyanmethemoglobin method. Mean corpuscular hemoglobin (MCH) was calculated as ratio of the hemoglobin concentration and the RBC count. The mean corpuscular hemoglobin concentration (MCHC) is the MCH divided by MCV. Hemoglobin F was quantified by the alkali denaturation test.[25]

Statistical analyses. Differences between neonatal and adult RBCs were tested using a two-tailed unpaired *t* test, and a *U* test was used when no normal distribution existed.[26] Three comparisons were made: (1) Comparison of the sample means; (2) Comparison of the entire neonatal and adult RBC populations (eg, 300 neonatal and 300 adult RBCs for most micropipette studies). The standard deviations of the populations reflect the spread of the individual RBC in all neonatal and adult samples and are therefore larger than the standard deviations of the sample means (Tables 1 and 2). (3) Comparison of the means of the 30 neonatal and 30 adult RBCs studied with the same micropipette. The number of degrees of freedom used for the first and second comparisons was based on the number of samples, whereas the number of degrees of freedom for the third comparison was based on the number of RBCs (ie, 30 in each sample). Linear regressions were calculated to determine correlations between two parameters measured for the same RBCs (ie, extensional modulus *v* bending modulus, and extensional modulus *v* extensional time constant). Significance of correlation coefficients was tested by using the number of RBCs studied in each group (ie, 298 degrees of freedom for the neonatal and for the adult RBCs).

RESULTS

Table 1 presents hematologic and cellular deformability data for neonatal and adult RBCs. The neonatal RBCs were larger (increased MCV, volume, resting cell diameter, and surface area) and contained larger amounts of fetal hemoglobin. The MCHC and the mean RBC thickness were similar for both types of cells.

The deformability of RBCs as measured by the rheoscope technique did not show significant differences between the neonates and the adults at any shear stress. However, the aspiration pressure (ie, negative pressure required to aspirate the whole RBCs completely into 3.3-μm diameter pipettes) for the average neonatal RBC was almost twice as high as that for the adult RBC (Table 1). Because the aspiration pressures for the entire RBC populations studied in each group (300 neonatal and 300 adult RBCs) did not demonstrate normal distributions, the cell populations were compared by using the *U* test in addition to the *t* test.[26] Both tests revealed significantly different aspiration pressures between each sample pair (RBCs from one neonate and one adult) studied with the same micropipette ($P < .05$).

Figure 1 shows the aspiration pressures of the 300 neonatal and 300 adult RBCs plotted against the volumes of the aspirated cells. The aspiration pressure of both cell types increased with increasing cell volume. However, the aspiration pressures of neonatal RBCs tended to be lower than that of adult RBCs when cells of same volume were compared. Similar relationships were found when the aspiration pressure was plotted against the resting cell diameter. No correlation existed between mean RBC thickness and aspiration pressure.

Table 2 presents viscoelastic material properties of neonatal and adult RBCs. The extensional (shear) elastic modulus (μ_e) of the neonatal RBCs was smaller by 10%, whereas the corresponding time constant (t_e) was 14% longer than that for the adult RBCs. Thus, the calculated membrane surface viscosity, computed as the product of μ_e times t_e, was similar for both cell types. There were significant ($P < .01$) negative correlations between μ_e and t_e for neonatal ($r = -.31$) and adult ($r = .20$) RBCs.

Bending characteristics also differed for the two groups (Fig 2 and Table 2). The suction pressure at which folding began was lower for neonatal RBCs than for adult RBCs when either 1- or 2-μm diameter pipettes were used (Fig 2). When these folding pressures and the appropriate cell diameters were used to calculate the bending modulus for the different cell types, it was found that the bending modulus of neonatal cells was lower by 16% compared with adult cells (Table 2); the same decrease was observed with 1- and 2-μm pipettes. There were significant ($P < .01$) correlations between the extensional and bending moduli measured in the 1-μm pipettes for neonatal ($r = .33$) and adult ($r = .28$) RBCs.

The viscosities of hemoglobin solutions with a concentration of 33 g/dL were similar for neonatal and adult RBCs (Tabel 2). The time constant for bending recovery (t_b) was computed as the product of hemoglobin viscosity times resting cell diameter divided by the bending modulus (see Materials and Methods, formula 10). Because the cell diameter was larger (Table 1) and the bending modulus was smaller (Table 2) for neonatal cells, the calculated t_b for neonatal RBCs was longer by 32% compared with adult cells. If t_b was related to the RBC diameter or to the surface area, then the mean difference was only 18% (Table 2).

DISCUSSION

Inspection of the present data leads to the following observations: (1) Neonatal RBCs require a higher suction pressure than adult RBCs for complete aspiration into the same pipette. (2) The membrane of neonatal RBCs is slightly less resistant to extensional and bending deformations. (3) Whole cell deformability as determined by the rheoscope and the hemoglobin and membrane surface viscosities are similar for neonatal and adult RBCs.

In this study, the suction pressure was slowly increased such that cellular dynamic properties did not meaningfully influence the pressure required to aspirate RBCs completely into 3.3-μm pipettes. Under these conditions, the aspiration pressure for a given pipette depends mainly on three factors: (1) the resting RBC diameter and surface area (ie, the necessary cellular deformation); (2) the minimum cylindrical diameter; (3) the static membrane elastic properties (ie, extensional and bending elastic moduli).

The first factor (ie, RBC diameter and surface area) most likely accounts for the increase in aspiration pressure with increasing RBC volume (Fig 1) and diameter and the higher aspiration pressure for neonatal RBCs (Table 1). Neonatal RBCs have to deform more than adult RBCs to enter the same pipette because of their wider diameter (Table 1) and their larger surface area.[20] Figure 1 may be misleading because it is the surface area and not the volume that determines the RBC deformation necessary for complete aspiration into a pipette. However, surface area cannot be estimated from the length of a cell folded into a 3.3-μm pipette. Therefore, we used RBC volume as an indicator of cellular surface area.

The smallest pipette that an RBC can enter without an increase in surface area or loss of volume is determined by its minimum cylindrical diameter. This minimal diameter is 3.0 μm for the average neonatal RBC and 2.8 μm for the average adult RBC.[20] Few neonatal RBCs failed to enter the 3.3-μm pipettes at an aspiration pressure of 500 dyn/cm^2 (Fig 1), thereby indicating that the minimum cylindrical diameter was seldom the limiting factor for RBC entry into these pipettes. However, the exponential increase in aspiration pressure with increasing cellular size suggests that the minimum cylindrical diameter of large RBCs approached the pipette diameter of 3.3 μm, thereby hindering their entry into the pipette unless high suction pressures were applied.

Entry of an RBC into a narrow micropipette begins with a smooth membrane extension, until the cell folds and enters the pipette completely. Thus, cell elongation (with attendant membrane extensional deformation) and folding occur when RBCs enter a 3.3-μm pipette. Both the extensional shear elastic modulus and the bending elastic modulus were slightly lower for neonatal cells than for adult RBCs (Table 2), ie, neonatal RBC membrane had lower resistance to extensional and folding deformation. This may explain why the aspiration pressures of neonatal cells tended to be lower than those of adult RBCs when cells with the same volume were compared (Fig 1).

RBCs are able to resume their original shape if the deforming forces are small and are applied for short periods.[14,17] The time constants for shape recovery from extensional and bending deformation increase as the corresponding elastic moduli decrease or as the membrane surface or hemoglobin viscosity increases.[14] This agrees with our observations of inverse relationships between the extensional time constant and extensional elastic modulus for both neonatal and adult RBCs. Moreover, the time constant for unfolding depends on the surface area of RBCs. We have, therefore, related this time constant to the surface area (if it was measured) or to the RBC diameter (Table 2). Both the resting diameter and the surface area of neonatal RBCs were approximately 12% larger when compared with adult RBCs (Table 1). The fact that neonatal cells have longer time constants due to their lower elastic moduli (Table 2) does not necessarily mean that they will take longer to achieve a given deformation. They would approach their final degree of deformation more slowly than adult cells, but since this final degree of deformation will be slightly greater for the neonatal cells, it is possible that at any time neonatal cells would be more greatly deformed than adult cells acted on by the same force.

Altered elastic moduli and time constants for shape recovery are caused by altered molecular structure of the cell membrane or cytoplasm.[3,14,27] Cross-linking of membrane skeleton proteins increases the membrane extensional elastic modulus,[3] whereas deficiency of spectrin or defective interaction of skeleton proteins decreases this modulus.[3] Such defects, as well as a decreased extensional elastic modulus, have been shown for membranes of RBC from patients with hereditary spherocytosis.[28,29] Neonatal and adult RBCs show no qualitative or gross quantitative differences in the pattern of membrane proteins,[30] and studies on the interaction of cytoskeleton proteins in membranes of neonatal RBCs do not appear to exist in the literature. However, spectrin is more difficult to extract from membranes of both neonatal RBCs and RBCs from patients with hereditary spherocytosis.[30,31] Whether this indicates that the decreased elastic modulus of neonatal RBCs has a similar basis as in hereditary spherocytosis is uncertain.

The molecular basis of bending rigidity (ie, bending elastic modulus) is unknown. A phospholipid bilayer has only bending rigidity and no extensional rigidity.[15] Hochmuth[32] speculates on a possible correspondence of the membrane surface viscosity and the apparent lipid viscosity determined by means of fluorescent probes. Perylene shows the same fluorescence polarization in membranes of neonatal and adult RBCs.[33] However, a study using diphenylhexatriene suggests that lipid viscosity is higher in neonatal RBCs than in adult cells.[34] The total amount of membrane lipids is increased in proportion to the larger membrane area of neonatal RBCs,[10,35] although the relative lipid composition of neonatal and adult RBCs is quite similar[35]; membranes of neonatal RBCs contain a greater proportion of saturated fatty acids.[35] However, given the equality of the calculated membrane viscosity for neonatal and adult RBCs (Table 2), this difference in fatty acid composition appears to be a minor determinant of this membrane mechanical property.

RBC deformability as determined by the rheoscope has been shown to depend mainly on the excess surface area, hemoglobin and membrane viscosity, and membrane extensional and bending elasticity[36-38]; RBC size is not important in this deformability assay.[9] The capability of "tank treading" is important for RBC deformation in the rheoscope. This ability depends on both membrane and cytoplasm viscosity.[36,38] Thus, the similar deformability of neonatal and adult RBCs in the rheoscope may be explained by similar membrane and cytoplasm viscosity.

Previous filtration studies have shown that neonatal RBCs filter less than adult RBCs.[4-6] Our finding of similar, or even lower, aspiration pressure of neonatal RBCs compared with adult RBCs with the same volume (Fig 1) and diameter indicates that the different filterability is not the result of decreased deformability, but of the larger size of neonatal RBCs. It is unlikely that the specific properties of neonatal RBCs observed in the present study (ie, increased aspiration pressure and prolonged rates of deformation) have major influences on the overall neonatal circulatory system, since the circulation in the neonate is characterized by low resistance and high flow conditions[39] and exchange transfusion with adult blood does not improve the circulation in the neonate.[40] Moreover, a recent study suggests that the lowest range of capillary diameters is at the limits of deformation of the circulating RBCs.[1] Thus, the diameter of the smallest capillaries in the neonate may be wider than in the adult, thereby compensating for the wider minimum cylindrical diameter of neonatal RBCs.

Our results indicate that the shortened life span of neonatal RBCs cannot be explained by decreased RBC deformability, as has been previously suggested.[4,5] Nevertheless, our findings may be relevant to the shortened life span of neonatal RBCs (60 to 80 days) compared with adult RBCs (120 days).[7] During aging, RBCs lose membrane fragments and water. This results in decreased surface area and in increased MCHC, cytoplasm, and membrane viscosity.[13] Continuous strain and immunologic mechanisms are probably involved in this steady fragmentation process. Eventually, these effete, poorly deformable RBCs will need longer times to traverse the reticuloendothelial system (eg, splenic pores), thereby aiding in their recognition and complete destruction by macrophages. Neonatal RBCs show accelerated loss of volume, surface area, and deformability compared with adult RBCs.[9] The increased pressure required for the large neonatal RBCs to enter 3.3-μm pipettes (Fig 1) suggests that the passage through splenic pores may also be more difficult. This may facilitate their fragmentation by immunologic mechanisms. Moreover, a decreased extensional elastic modulus suggests that the membrane is not only more flexible, but also more prone to yield and rupture in response to a given force[3,29]; this agrees with the findings of decreased mechanical resistance of neonatal RBCs.[21] Thus, both impaired ability of neonatal RBCs to pass the narrow splenic pores and decreased resistance to fragmentation may contribute to the shortened life span of neonatal RBCs.

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REFERENCES

1. Canham PB, Potter RF, Woo D: Geometric accommodation between the dimensions of erythrocytes and the calibre of heart and muscle capillaries in the rat. J Physiol 347:697, 1984

2. Schmid-Schönbein H: Microrheology of erythrocytes, blood viscosity and the distribution of blood flow in the microcirculation. Int Rev Physiol 9:1, 1976

3. La Celle PL, Evans EA, Hochmuth RM: Erythrocyte membrane elasticity, fragmentation and lysis. Blood Cells 3:335, 1977

4. Gross GP, Hathaway WE: Fetal erythrocyte deformability. Pediatr Res 6:593, 1972

5. Tillmann W, Zabel U, Lakomek M, Schröter W: Influence of the fluidity of hemoglobin F on the flexibility of red cells of newborn and older infants. Bibl Anat 20:222, 1980

6. Reinhart WH, Danoff SJ, King RG, Chien S: Rheology of fetal and maternal blood. Pediatr Res 19:147, 1985

7. Bratteby LE, Garby L, Groth T, Schneider W, Wadman B: Studies on erythro-kinetics in infancy. XII. The mean life span and the life span frequency function of red blood cells formed during foetal life. Acta Paediatr Scand 57:311, 1968

8. Linderkamp O, Versmold HT, Riegel KP, Betke K: Contributions of red cells and plasma to blood viscosity in preterm and full-term infants and adults. Pediatrics 74:45, 1984

9. Linderkamp O, Wu PYK, Meiselman HJ: Deformability of density separated red blood cells in normal newborn infants and adults. Pediatr Res 16:964, 1982

10. Coulombel L, Tchernia G, Feo C, Mohandas N: Echinocytic sensitivity and deformability of human newborn red cells. Biol Neonate 42:284, 1982

11. La Celle PL: Pathologic erythrocytes in the capillary microcirculation. Blood Cells 1:269, 1975

12. Meiselman HJ: Morphological determinants of red cell deformability. Scand J Clin Lab Invest 41:27, 1981 (suppl 156)

13. Linderkamp O, Meiselman HJ: Geometric, osmotic, and membrane mechanical properties of density-separated human red cells. Blood 59:1121, 1982

14. Evans E, Mohandas N, Leung A: Static and dynamic rigidities of normal and sickle erythrocytes. J Clin Invest 73:477, 1984

15. Evans EA: Bending elastic modulus of red blood cell membrane derived from buckling instability in micropipet aspiration tests. Biophys J 43:27, 1983

16. Evans EA, La Celle PL: Intrinsic material properties of the erythrocyte membrane indicated by mechanical analysis deformation. Blood 45:29, 1975

17. Hochmuth RM, Worthy PR, Evans EA: Red cell extensional recovery and the determination of membrane viscosity. Biophys J 26:101, 1979

18. Smith CM II, Kuettner JF, Tukey DP, Burris SM, White JG: Variable deformability of irreversibly sickled erythrocytes. Blood 58:71, 1981

19. Smith CM II, Tukey DP, Krivit W, White JG: Fetal red cells (FC) differ in elasticity, viscosity, and adhesion from adult red cells (AC). Pediatr Res 15:588, 1981 (abstr)

20. Linderkamp O, Wu PYK, Meiselman HJ: Geometry of neonatal and adult red blood cells. Pediatr Res 17:250, 1983

21. Sjölin S: The resistance of red cells in vitro. A study of the osmotic properties, the mechanical resistance and the storage behaviour of red cells of fetuses, children and adults. Acta Pediatr 43:1, 1954 (suppl 98)

22. Cokelet GR, Bush RW, La Celle PL: Electroviscous flow and electrophoretic motion during erythrocyte entry in a glass capillary in the presence of applied electric potential gradients. Pflugers Arch 390:94, 1981

23. Chien S, Sung KLP, Skalak R, Usami S, Tözeren A: Theoretical and experimental studies on viscoelastic properties of erythrocyte membrane. Biophys J 24:463, 1978

24. Cokelet GR, Meiselman HJ: Rheological comparison of hemoglobin solutions and erythrocyte suspensions. Science 162:275, 1968

25. Betke K, Marti H, Schlicht I: Estimation of small percentage of foetal haemoglobin. Nature 184:1877, 1959

26. Campbell RC: Statistics for biologists. London, Cambridge University Press, 1967

27. Nash GB, Johnson CS, Meiselman HJ: Mechanical properties of oxygenated red blood cells in sickle cell (HbSS) disease. Blood 63:73, 1984

28. Goodman SR, Shiffer KA, Casoria LA, Eyster ME: Identification of the molecular defect in the erythrocyte membrane skeleton of some kindreds with hereditary spherocytosis. Blood 60:772, 1982

29. Waugh RE, La Celle PL: Abnormalities in the membrane material properties of hereditary spherocytes. J Biomech Eng 102:240, 1980

30. Shapiro DL, Pasqualini P: Erythrocyte membrane proteins of premature and full-term newborn infants. Pediatr Res 12:176, 1978

31. Landaw SA, Rathbun SC, Guancial RL: Evidence for a tightly-linked spectrin lattice in red blood cells (RBC) of newborn (NB) man. Pediatr Res 16:208A, 1982 (abstr)

32. Hochmuth RM: Viscoelastic solid behavior of red cell membrane, in Cokelet GR, Meiselman HJ, Brooks DE (eds): Erythrocyte Mechanics and Blood Flow. New York, Alan R Liss, 1980, p 57

33. Kehry M, Yguerabide J, Singer SJ: Fluidity in the membranes of adult and neonatal human erythrocytes. Science 195:486, 1977

34. Crespo LM, Bifano EM, Freedman JC: Membrane lipid fluidity and filterability of human RBCs from adults and newborns. Pediatr Res 18:239A, 1984 (abstr)

35. Neerhout RC: Erythrocyte lipids in the neonate. Pediatr Res 2:172, 1968

36. Schmid-Schönbein H, Gaethgens P: What is red cell deformability? Scand J Clin Lab Invest 41:13, 1981 (suppl 156)

37. Pfafferott C, Wenby R, Meiselman HJ: Morphologic and internal viscosity aspects of RBC rheologic behavior. Blood Cells 8:65, 1982

38. Trans-Son-Tay R, Sutera SP: Determination of RBC membrane viscosity from rheoscopic observations of tank-treading motion. Biophys J 46:65, 1984

39. Linderkamp O, Strohhacker I, Versmold HT, Klose H, Riegel KP, Betke K: Peripheral circulation in the newborn: Interaction of peripheral blood flow, blood pressure, blood volume, and blood viscosity. Eur J Pediatr 129:73, 1978

40. Fouron JC, Bard H, Riopel L, Muylder X, Van Ameringen MR, Urfer F: Circulatory changes in newborn lambs with experimental polycythemia: Comparison between fetal and adult type blood. Pediatrics 75:1054, 1985

A Chromosomal Breakage Syndrome With Profound Immunodeficiency

By Mary Ellen Conley, Nancy B. Spinner, Beverly S. Emanuel, Peter C. Nowell, and Warren W. Nichols

The chromosomal breakage syndromes—ataxia-telangiectasia, Fanconi's anemia, and Bloom's syndrome—are associated with growth failure, neurologic abnormalities, immunodeficiency, and an increased incidence of malignancy. The relationship between these features is unknown. We recently evaluated a 21-year-old female with more severe chromosomal breakage, immunodeficiency, and growth failure than in any of the mentioned disorders. As of November 1985, the patient remains clinically free of malignancy. At age 18, the patient's weight was 22.6 kg (50th percentile for seven years), height was 129 cm (50th percentile for eight years), and head circumference was 42 cm (50th percentile for six months). Laboratory studies demonstrated a marked decrease in both B and T cell number and function. The peripheral blood contained 400 to 900 lymphocytes/μL with 32% T_{11} cells, 17% T_4 cells, and 21% T_8 cells. The proliferative responses to phytohemagglutinin (PHA), pokeweed mitogen, and concanavalin A were less than 10% of control. There were 1% surface IgM positive cells, and serum IgG was 185 mg/dL, IgM 7 mg/dL, IgA 5 mg/dL. In lymphocyte cultures stimulated with the T cell mitogens PHA, phorbol ester, and interleukin 2, 55% of the banded metaphases demonstrated breaks or rearrangements. The majority of the breaks involved four fragile sites on chromosomes 7 and 14, 7p13, 7q35, 14q11, and 14q32. These are the sites of the genes for the T cell-antigen receptor and the immunoglobulin heavy chain and are sites of gene rearrangement in lymphocyte differentiation. Epstein-Barr virus stimulated B cells and fibroblast cultures also demonstrated a high incidence of breaks, but the sites were less selective. These findings suggest that the sites of chromosomal fragility in the chromosomal breakage syndromes may be informative and that factors other than the severity of the immunodeficiency or the high incidence of chromosomal damage may contribute to the occurrence of malignancy in the chromosomal breakage syndromes.

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THE CHROMOSOMAL BREAKAGE SYNDROMES—ataxia-telangiectasia,[1-4] Fanconi's anemia,[5-7] and Bloom's syndrome[8-11]—have several features in common. All are disorders inherited in an autosomal recessive pattern in which spontaneous chromosomal breaks, gaps, and rearrangements are seen in mitogen-stimulated T cells. Although each syndrome can be characterized by distinct clinical and laboratory findings, all have been associated with growth failure, microcephaly, neurologic abnormalities, immunodeficiency, failure to develop secondary sexual characteristics, and an increased incidence of malignancy. The relationship between these features is unclear. It has been postulated that the increased incidence of malignancy may be related to inadequate immune surveillance or immune deficiency.[1,3,7] Alternatively, the spontaneous chromosomal breaks may result in an increased rate of mutation, which in turn may result in malignancy.[5,8,9] We have recently studied a 21-year-old patient who does not have one of the well-described chromosomal breakage syndromes, who has significant chromosomal instability characterized by spontaneous breaks, gaps, and rearrangements involving multiple chromosomes in T cells, Epstein-Barr virus (EBV)-transformed B cell lines, and cultured fibroblasts. In the T cells, the breaks selectively involve four fragile sites on chromosomes 7 and 14 as has been seen in ataxia-telangiectasia.[4,12,13] In the B cell lines and fibroblasts, the abnormalities are less selective. In addition, the patient has profound immunodeficiency involving both B and T cells. At present, the patient has had no malignancies, and there is no family history suggesting an unusual predisposition to malignancy.

CASE REPORT

D.M. was the 5 lb 6 oz product of a full-term uncomplicated pregnancy delivered without problems to a 35-year-old primagravida. Both parents were of eastern European ancestry without consanguinity for at least four generations. The patient was noted to have poor growth and microcephaly within the first three months of life. Between 4 and 6 years of age, the patient developed a chronic cough. Hypogammaglobulinemia was diagnosed at 6 years of age and the patient has been treated with immunoglobulin replacement since that time. At 8 years of age, she was evaluated for short stature. She was noted to be lymphopenic with decreases in both B and T cell numbers; proliferative responses to mitogens were poor. Serum IgG was 120 mg/dL, IgM was 20 mg/dL, and IgA was undetectable. Karyotype analysis was difficult because of poor T cell proliferation but suggested a constitutional abnormality of chromosome 7. The patient was lost to follow-up but continued to receive intramuscular gammaglobulin. In 1983, at age 18, the patient was referred to Children's Hospital of Philadelphia for evaluation of hypogammaglobulinemia. In the preceding ten years, the patient had had poor growth and a persistent cough but had not required hospitalization for infection or other problems. She had no history of skin rashes, sun sensitivity, telangiectasia, ataxia, or anemia. She had not reached menarche. The patient had completed sophomore year in a school for learning disabled children. Both parents appeared to be of dull normal intelligence; there were no other children. The patient's maternal grandfather may have died of cancer at age 66. There was no other history of malignancy, unusual infections, or fetal wastage in more than 40 first- and second-degree relatives of the patient and her parents.

On physical examination, the patient was a pale, small, pleasant girl with marked microcephaly. Height was 129 cm (<3rd percentile, 50th percentile for 8-year-old), weight 22.6 kg (<3rd percentile, 50th percentile for 7-year-old), and head circumference 42 cm (<3rd percentile, 50th percentile for 6-month-old). The skin was pale and doughy without rashes, telangiectasia, or areas of increased

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or decreased pigmentation. The facies were birdlike, with a prominent, beaked nose and recessed chin. The ears were low, and there was hypoplasia of the dental enamel. The tympanic membranes appeared scarred. The tonsils were small, and there was no cervical lymphoid tissue palpable. Chest examination revealed bilateral rales. There were no murmurs or unusual heart sounds. The liver and spleen were not felt. There was scant straight pubic hair and no breast tissue. Neurologic examination was normal.

On complete blood count, the patient had a hemoglobin of 14.6 g/dL and a hematocrit of 43% with normal red cell indices, WBC was 14,400/μL with 82% neutrophils, 5% bands, 5% lymphocytes, 1% eosinophils, and 7% monocytes. Platelet count was 575,000/μL. IgG was 185 mg/dL (on gammaglobulin therapy), IgM was 7 mg/dL, and IgA was less than 5 mg/dL. Chest x-ray demonstrated bibasilar infiltrates with increased interstitial markings throughout both lung fields and peribronchial thickening. She had bilateral opacification of maxillary and frontal sinuses. Alpha-fetoprotein level was normal at 10 ng/mL. Antinuclear antibodies, anti-red cell antibodies, and antibodies to thyroid, smooth muscle, and mitochondria were not present. Somatomedin C was normal. Follicle-stimulating hormone was 40 mIU/mL and luteinizing hormone was 50 mIU/mL. These values are consistent with an ovulatory phase or postmenopausal phase but not normal premenarche.

MATERIALS AND METHODS

Peripheral blood lymphocytes were obtained from heparinized venous blood by Ficoll-Hypaque centrifugation. To assess proliferation in response to standard mitogens, lymphocytes were cultured at 0.5×10^5 cells per milliliter in round-bottom microtiter wells for 72 hours with 5% phytohemagglutin (PHA) (GIBCO, Grand Island, NY), 5% pokeweed mitogen (GIBCO), or 30 μg/mL concanavalin A (DIFCO, Detroit) and 15% heat-inactivated human serum. Four hours before the cells were harvested, 1 μCi ^{3}H-thymidine was added to each well. All cultures were run in triplicate. Immunofluorescence staining of lymphocytes was performed as previously described.[14] Cells were enumerated with a fluorescence microscope equipped with epi-illumination (Carl Zeiss, Oberkochen, FRG).

Metaphase chromosomes were obtained from peripheral blood lymphocytes stimulated with PHA or with a mixture of T cell mitogens (PHA, phorbol ester, and interleukin 2[15]). All fragility studies were conducted on cells harvested after 48 hours. To assess bleomycin sensitivity, 30 μg/mL of bleomycin was added 2.5 hours before the end of the culture period. Sister chromatid exchange rates in the presence and absence of mitomycin C (25 ng/mL) were determined by standard methods.[16] X-ray–induced chromosomal aberrations were studied by irradiating lymphocytes at three dose levels—100 rad, 200 rad, and 400 rad—before PHA stimulation.

Permanent cell lines from fibroblasts (GM7166) and B lymphocytes (GM7078) were established by the mutant cell repository (Institute for Medical Research, Camden, NJ).

RESULTS

Immunologic studies. The patient has persistent lymphopenia with absolute lymphocyte counts between 400 and 900/μL first noted in early childhood. Lymphocyte enumeration, using indirect immunofluorescence staining techniques, demonstrated marked reduction of all lymphocyte subsets (Table 1). Cells bearing the T cell helper phenotype (T_4) were more reduced in number than cells expressing the T cell cytotoxic–suppressor phenotype (T_8), resulting in a reversal of the normal T_4-T_8 ratio (0.8 in the patient compared with 1.6 ± 0.3 in healthy laboratory controls). B cells could be detected with a monoclonal anti-B cell antibody, B1, or with fluorescein-conjugated antihuman IgM. Further analysis of the B cell phenotype using two-color immunofluorescence staining demonstrated that all of the cells expressing surface IgM also expressed surface IgD; 52% of the B cells could be indirectly stained with the monoclonal antibody B2, which binds to the EBV receptor.[17] In contrast, 89% ± 6% of B cells from controls express the B2 marker.[14] Kappa-lambda ratio was also abnormal. Eighty percent of the patient's B cells expressed kappa and 20% expressed lambda light chain. In healthy controls, B cells are almost equally divided between cells bearing kappa and cells bearing lambda.[14] The patient's lymphocytes did proliferate in response to standard mitogen stimulation, but the response was less than 10% of the control assayed concurrently.

Table 1. Immunologic Studies

	Patient		Controls (Mean ± 1 SD)	
	%	Absolute Number	%	Absolute Number
Total lymphocytes		600		2,300 ± 630
T cells (T_{11})	32	192	67 ± 9	1,580 ± 220
T helpers (T_4)	17	102	46 ± 6	1,058 ± 190
T suppressors (T_8)	21	126	26 ± 6	598 ± 150
B cells (B_1)	1	6	11 ± 3	253 ± 81
Surface IgM	1	6	9 ± 3	207 ± 77
Mitogen responses*				
PHA		806		49,927 ± 28,076
Pokeweed		698		16,961 ± 9,267
ConA		613		17,637 ± 10,658
Background		107		252 ± 306

*Expressed as cpm ^{3}H-thymidine.

Cytogenetics studies. Chromosome analysis of the proband's peripheral T lymphocytes on unbanded chromosomes revealed a high level of spontaneous aberrations, with 38% of the metaphases having abnormalities, including breaks, rings, dicentrics, acentric fragments, and translocations (Table 2). The incidence of aberrant metaphases was even higher in Giemsa-banded material. Of 81 cells karyotyped, 29 had rearrangements involving chromosome 7 or 14. Another 16 cells had rearrangements that appeared to be random but did not involve chromosome 7 or 14, for a total of 55% aberrant metaphases.

In the cells with rearrangements of chromosome 7 or 14, there was clustering of breakpoints at 7p13, 7q35, 14q11, and 14q32 (Fig 1). Among these were three cells with inv(7)(p13q35), four with inv dup(7)(q35q11), three with t(7;14)(q34q11.2), and a small clone of eight cells with t(14;14)(q11;q32) (Fig 2).

Treatment of the patient's cells with bleomycin during the G_2 phase of the cell cycle resulted in an increase in chromosome aberrations, as compared with controls (Table 2). A similar increase has been reported in cells from patients with ataxia-telangiectasia.[18] Comparable studies, with and without bleomycin, were carried out on the patient's parents. As seen in Table 2, there was no increase in spontaneous fragility in the parents, but the data suggest increased sensitivity to bleomycin, as is seen in obligate heterozygote carriers of ataxia-telangiectasia.

Table 2. Frequency of Aberrations in Unstressed Lymphocytes

	Total Metaphases	Metaphases with Aberrations (%)	Breaks (breaks/cell)	Complex Rearrangements	Total No. of Aberrations (per cell)	Chromatid Gaps
Patient	73	28(38.3%)	13(.18)	14(.19)	27(.37)	6(.08)
Mother	101	5(5.0%)	5(.05)	0(-0-)	5(.05)	9(.09)
Father	164	9(5.5%)	9(.06)	1(.01)	10(.06)	15(.09)
Control	100	2(2.0%)	2(.02)	0(-0-)	2(.02)	2(.02)
	Frequency of aberrations in lymphocytes exposed to bleomycin for 2.5 hours in G_2					
Patient	88	75(85%)	207(2.4)	12(.14)	219(2.5)	83(.94)
Mother	103	47(46%)	125(1.2)	0(-0-)	125(1.2)	80(.78)
Father	118	54(46%)	114(.97)	1(.01)	115(.97)	66(.56)
Control	100	22(22%)	28(.28)	0(-0-)	28(.28)	26(.26)

The control was studied concurrently with the patient. In controls studied in our laboratory, the range for breaks/cell in unstressed lymphocytes was from 0 to 0.07. When control lymphocytes were stressed with bleomycin, the range was from 0.28 to 0.71 breaks/cell.

Sister chromatid exchange (SCE) studies in the presence and absence of mitomycin C were done on peripheral lymphocytes from the proband and her parents. SCE levels in all instances were within the expected range for our laboratory.

To examine chromosomal fragility in B cells, EBV-stimulated B cell cultures were studied on three occasions in two laboratories. A total of 178 metaphases were examined, and in all instances, there was a high frequency of metaphases with aberrations, ranging from 26% to 50%. Alterations in chromosomes 7 and 14 were found in 12% of banded metaphases but usually involved terminal associations, producing dicentric chromosomes, rather than the nonrandom breakpoints observed in the T cell cultures. SCE studies on the B cell cultures, with or without mitomycin C, were within normal limits for the laboratory.

A fibroblast culture from the patient was established. Of 15 metaphases that were karyotyped, five demonstrated rearrangements (33%), and none of these involved chromosomes 7 and 14. SCE studies were again normal.

The effect of x-irradiation on the cells of the patient and her parents were studied. There was no evidence of increased sensitivity in the parents' peripheral blood T cell cultures or in their long-term B cell lines. The T cells, B cells, and fibroblasts from the patient showed elevated levels of chromosomal breakage on exposure to x-irradiation, but when corrected for spontaneous breakage, the degree of damage was within normal limits. However, there was increased sensitivity to cell killing induced by x-irradiation. At the highest dose (400 rad), mitotic activity was virtually eliminated from the T cell cultures. A more detailed study of the x-ray sensitivity of the patient's fibroblasts, and comparison with the findings in ataxia-telangiectasia, will be published separately.[18a]

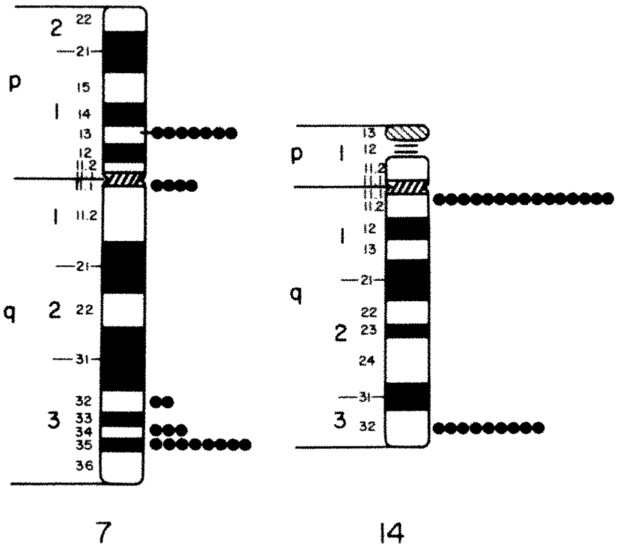

Fig 1. Karyotype of D.M. with 46,XX t(14;14) (q11;q32). Inset demonstrates another example of this translocation.

DISCUSSION

Although the patient described in this report has several of the findings seen in all the chromosomal breakage syndromes, she does not have the cardinal features seen in any one of these disorders. She does not have the rash or sun sensitivity seen in Bloom's syndrome, the telangiectasia or ataxia characteristic of ataxia-telangiectasia, or the pancytopenia or bony abnormalities typical of Fanconi's anemia. Her DNA repair defects are most similar to those seen in ataxia-telangiectasia.[18-20] However, her immunodeficiency and spontaneous chromosomal breakage are more profound than that seen in any of the well-described syndromes.

The immunologic defects in each of the chromosomal breakage syndromes are variable and may progress with age. The best documented and most consistent abnormalities are seen in ataxia-telangiectasia in which serum IgA and IgE are usually absent, lymphocyte numbers, particularly T cells, are decreased, and proliferative responses to mitogens are decreased to a mean of 60% of normal.[2,3] Patients with Bloom's syndrome or Fanconi's anemia may have mildly decreased concentrations of serum immunoglobulins, but proliferative responses to PHA are usually normal.[7,10,11] In contrast, our patient has markedly decreased T cell numbers and a proliferative response to mitogens that is less than 10% of control. She also has panhypogammaglobulinemia and low numbers of B cells. The B cells that are present express an immature phenotype similar to that seen in X-linked agammaglobulinemia.[14]

The degree of chromosomal breakage in each of the three syndromes is also variable and is influenced by culture and

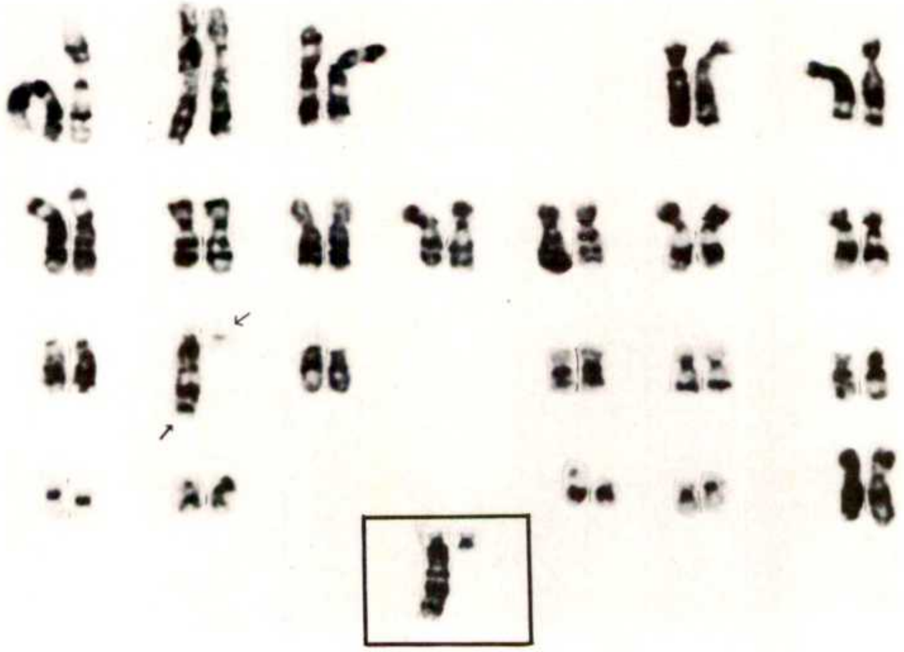

Fig 2. Schematic diagram of chromosomes 7 and 14, with each circle indicating the involvement of a specific site in a chromosomal rearrangement.

assay techniques and the origin of the cells examined. However, there are specific cytogenetic findings typical for each syndrome. Lymphocytes from patients with ataxia-telangiectasia are hypersensitive to x-irradiation and to the radiomimetic agent bleomycin,[18,19] and often demonstrate cytogenetically abnormal clones, frequently involving chromosomes 7 and 14.[4,12,13] Bloom's syndrome is characterized by an increase in quadriradial figures and an increase in spontaneous SCEs.[20] In Fanconi's anemia, there are increased spontaneous breaks and increased breaks and radial figures after exposure to DNA cross-linking agents, such as mitomycin C.[21] By cytogenetic studies, our patient is most similar to patients with ataxia-telangiectasia. She has increased spontaneous chromosome aberrations in lymphocyte cultures, abnormal chromosomes, including translocations and dicentrics, increased sensitivity to bleomycin, and an apparently abnormal clone involving both chromosomes 14. She has normal levels of SCE and does not have increased levels of quadriradial exchange figures. However, there are differences between our patient and patients with ataxia-telangiectasia. EBV-stimulated B cell lines from patients with ataxia-telangiectasia have not demonstrated increased chromosomal breakage,[4,13,22] whereas 26% to 50% of the metaphases from our patient's B cell lines were abnormal.

In patients with ataxia-telangiectasia, in our patient, and in other patients in whom the site of chromosomal breakage has been carefully examined, chromosomal breaks in mitogen-stimulated T cells selectively involve four sites.[4,12,13,23] At least three of these four locations—7q35, 14q11, and 14q32—are now recognized as sites of gene rearrangement during lymphocyte differentiation. The genes for the β and α chain of the T cell-antigen receptor are at 7q35 and 14q11, respectively.[24-27] The immunoglobulin heavy chain gene is at 14q32.[28] Preliminary evidence suggests that the fourth frequently involved fragile site, 7p13, is the site of a gene showing homology to the T cell receptor β chain gene and may be the T cell receptor γ chain gene.[27] One might expect other sites of chromosomal fragility in cell lines not using the antigen receptor genes. Accordingly, chromosome breaks in fibroblast cell lines from our patient and patients with ataxia-telangiectasia do not selectively involve chromosomes 7 and 14.[13] Current tissue culture techniques permit the long-term growth and proliferation of a variety of cell types, including melanocytes, glial cells, and thymic epithelial cells. Cell lines from patients like ours may demonstrate tissue-specific chromosome fragility sites, marking genes of particular importance in that cell line.

Variants of the chromosomal breakage syndromes have been described. These have usually been in patients who demonstrate some but not all of the clinical features of a particular syndrome. Webster et al[29] described a patient similar to ours, an 18-year-old girl with growth failure, chronic pulmonary disease, selective IgA deficiency, lymphopenia with poor proliferative responses to mitogens, and defective DNA repair. However, spontaneous chromosomal breakage was not described. A group from the University of Nijmegen, in The Netherlands, has reported findings on two brothers with microcephaly, growth retardation, mental retardation, café au lait spots, dysgammaglobulinemia, and chromosomal instability, particularly involving chromosomes 7 and 14.[23,30] These boys may have the same disorder as our patient, although our patient has more profound abnormalities of the immune system on laboratory testing and more profound growth failure. Seemanova et al[31] also described a group of patients with a disorder resembling that seen in our patient. These nine patients had microcephaly with normal intelligence, immunodeficiency, and an increased incidence of malignancy. Although the authors state that chromosome instability did not appear to be a problem, they also note that "chromosome analysis was repeatedly unsuccessful owing to failure of blast transformation."

The well-described chromosomal breakage syndromes are inherited in an autosomal recessive pattern. Lymphocytes from both parents of our patient appeared to have increased sensitivity to chromosomal damage by bleomycin, suggesting a similar pattern of inheritance. Of interest, the patient's mother has IgA deficiency (<5 mg/dL) and borderline microcephaly (head circumference 53 cm).

A feature that makes our patient of particular interest is that despite her profound immunodeficiency and her high incidence of chromosomal breakage, she has not developed a malignancy. The increased frequency of malignancy in the chromosomal breakage syndromes, which usually occurs before age 20, has been attributed to both the immunodeficiencies associated with these disorders and the chromosomal breaks.[1,3,5,7-9] Our patient's relatively benign clinical course suggests that the equation may be more complex. Mice treated with irradiation at high dose rate develop a greater number of chromosomal breaks in liver cells than do mice irradiated at low dose rate. However, the incidence of hepatoma and ovarian adenoma is higher in animals irradiated at low dose rate.[32] The extensive chromosomal damage after irradiation at high dose rate may render many potential cancer cells incapable of mitosis. A similar phenomenon may be occurring in our patient, with her marked chromosomal fragility eliminating some potentially neoplastic cells, and perhaps also contributing to the severe degree of immunodeficiency and growth retardation. Additional evidence concerning these speculations may be provided by the subsequent history of the small clone of T cells with a t(14;14)(q11;q32) translocation, now present in her peripheral blood (Fig 2). In ataxia-telangiectasia, progression of such clones to T cell leukemia, with additional karyotypic changes, has been described.[4]

The numerous questions raised by the study of this patient indicate the need for more multidisciplinary investigations of such atypical "fragility syndromes." Not only may additional specific disorders be precisely defined, but also cells from such patients can provide an important contribution that will supplement material from the classic "breakage syndromes" in the study of specific genes and chromosomal mechanisms involved in normal and abnormal growth regulation.

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REFERENCES

1. Boder E: Ataxia-telangiectasia: Some historic, clinical and pathologic observations. Birth Defects: Original Article Series 9:255, 1975

2. Gatti RA, Bick M, Tam CF, Medici MA, Oxelius VO, Holland M, Goldstein AL, and Boder E: Ataxia-telangiectasia: A multiparameter analysis of eight families. Clin Immunol Immunopathol 23:501, 1982

3. Waldmann TA, Misiti J, Nelson DL, Kraemer KH: Ataxia-telangiectasia: A multisystem hereditary disease with immunodeficiency, impaired organ maturation, X-ray hypersensitivity, and a high incidence of neoplasia. Ann Intern Med 99:367, 1983

4. Kaiser-McCaw B, Hecht F: Ataxia-telangiectasia; chromosomes and cancer, in Bridges BA, Harnden DG (eds): Ataxia-Telangiectasia—A Cellular and Molecular Link Between Cancer, Neuropathology and Immune Deficiency. New York, Wiley, 1982, p 243

5. Bloom GE, Warner S, Gerald PS, Diamond LK: Chromosome abnormalities in constitutional aplastic anemia. N Engl J Med 274:8, 1966

6. Swift MR, Hirschhorn K: Fanconi's anemia, inherited susceptibility to chromosome breakage in various tissues. Ann Intern Med 65:496, 1966

7. Pedersen FK, Hertz H, Lundsteen C, Platz P, Thomsen M: Indication of primary immune deficiency in Fanconi's anemia. Acta Paediatr Scand 66:745, 1977

8. Sawitsky A, Bloom D, German J: Chromosomal breakage and acute leukemia in congenital telangiectatic erythema and stunted growth. Ann Intern Med 65:487, 1966

9. German J: Bloom's syndrome. I. Genetical and clinical observations in the first twenty-seven patients. Am J Hum Genet 21:196, 1969

10. Schoen EJ, Shearn MA: Immunoglobulin deficiency in Bloom's syndrome. Am J Dis Child 113:594, 1967

11. Hutteroth TH, Litwin SD, German J: Abnormal immune response of Bloom's syndrome lymphocytes in vitro. J Clin Invest 56:1, 1975

12. Scheres JMJC, Hustinx TWJ, Weemaes CMR: Chromosome 7 in ataxia-telangiectasia. J Pediatr 97:440, 1980

13. O'Connor RD, Brown MG, Francke U: Immunologic and karyotypic studies in ataxia-telangiectasia: Specificity of break points on chromosomes 7 and 14 in lymphocytes from patients and relatives, in Bridges BA, Harnden DG (eds): Ataxia-Telangiectasia—A Cellular and Molecular Link Between Cancer, Neuropathology and Immune Deficiency. New York, Wiley, 1982, p 259

14. Conley ME: B Cells in patients with X-linked agammaglobulinemia. J Immunol 134:3070, 1985

15. Conley ME, Nowell PC, Henle G, Douglas SD: XX T cells and XY B cells in two patients with severe combined immune deficiency. Clin Immunol Immunopathol 31:87, 1984

16. Latt SA: Sister chromatid exchange formation. Ann Rev Genet 15:11, 1981

17. Fingeroth JD, Weis JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT: Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc Natl Acad Sci USA 81:4510, 1984

18. Cramer P, Painter RB: Bleomycin-resistant DNA synthesis in ataxia-telangiectasia cells. Nature 291:671, 1981

19. Cohen MM, Simpson SJ, Pazos L: Specificity of bleomycin-induced cytotoxic effects on ataxia-telangiectasia lymphoid cell lines. Cancer Res 41:1817, 1981

20. German J, Schonberg S, Louie E, Chaganti RSK: Bloom's syndrome. IV. Sister chromatid exchanges in lymphocytes. Am J Hum Genet 29:248, 1977

21. Sasaki MS, Tonomura A: A high susceptibility of Fanconi's anemia to chromosomal breakage by DNA cross-linking agents. Cancer Res 33:1829, 1973

22. Cohen MM, Sagi M, Ben-Zur Z, Schaap T, Voss R, Kohn G, and Ben-Bassat H: Ataxia-telangiectasia: Chromosomal stability in

continuous lymphoblastoid cell lines. Cytogenet Cell Genet 23:44, 1979

23. Hustinx TWJ, Scheres JMJC, Weemaes CMR, Haar BGA, Janssen AH: Karyotype instability with multiple 7/14 and 7/7 rearrangements. Hum Genet 49:199, 1979

24. Croce CM, Isobe M, Palumbo A, Puck J, Ming J, Tweardy D, Erikson J, Davis M, Rovera G: Gene for α-chain of human T-cell receptor: Location on chromosome 14 region involved in T-cell neoplasms. Science 227:1044, 1984

25. Jones C, Morse HG, Kao FT, Carbone A, Palmer E: Human T-cell receptor α-chain genes: Location on chromosome 14. Science 228:83, 1985

26. Isobe M, Erikson J, Emanuel BS, Nowell PC, Croce CM: Location of gene for β subunit of human T-cell receptor at band 7q35, a region prone to rearrangements in T cells. Science 228:580, 1985

27. Morton CC, Duby AD, Eddy RL, Shows TB, Seidman JG: Genes for β chain of human T-cell antigen receptor map to regions of chromosomal rearrangement in T cells. Science 228:582, 1985

28. Kirsch IR, Morton CC, Nakahara K, Leder P: Human immunoglobulin heavy chain genes map to a region of translocation in malignant B lymphocytes. Science 216:301, 1982

29. Webster D, Arlett CF, Harcourt SA, Teo I, Henderson L: A new syndrome of immunodeficiency and increased cellular sensitivity to DNA damaging agents, in Bridges BA, Harnden DG (eds): Ataxia-Telangiectasia—A Cellular and Molecular Link Between Cancer, Neuropathology and Immune Deficiency. New York, Wiley, 1982, p 379

30. Weemaes CMR, Hustinx TWJ, Scheres JMJC, Van Munster PJJ, Bakkeren JAJM, Taalman RDFM: New chromosomal instability disorder: The Nijmegen breakage syndrome. Acta Paediatr Scand 70:557, 1981

31. Seemanova E, Passarge E, Beneskova D, Houstek J, Kasal P, Sevcikova M: Familial microcephaly with normal intelligence, immunodeficiency, and risk for lymphoreticular malignancies: A new autosomal recessive disorder. Am J Med Genet 20:639, 1985

32. Cole LJ, Nowell PC: Radiation Carcinogenesis: The sequence of events. Science, 150:1782, 1965

Human Macrophage Maturation and Heterogeneity: Analysis With a Newly Generated Set of Monoclonal Antibodies to Differentiation Antigens

By Reinhard Andreesen, Klaus J. Bross, Jürgen Osterholz, and Frank Emmrich

We have analyzed the expression of late differentiation antigens during terminal in vitro maturation of human macrophages (MØ) from blood monocytes (MO) in comparison to their distribution among mature MØ residing in various tissue sites. By immunizing mice with MØ derived from blood MO by culture on hydrophobic Teflon foils, monoclonal antibodies (mAbs) were developed (MAX.1, MAX.2, MAX.3, MAX.11) that reacted with lineage-restricted differentiation antigens. These antigens were expressed exclusively on MØ or were markedly increased after in vitro differentiation. The only overlap to another hemopoietic cell lineage was observed with MAX.3, which is shared by platelets and megakaryocytes. In the course of MØ maturation in vitro, the MAX.1 and MAX.3 antigens are detected within the cytoplasm two days before they appear on the cell surface. In contrast, the MAX.11 antigen is expressed simultaneously in the cytoplasm and at the cell surface, is found in varying degrees on a minor portion of blood MO and U937 cells, and is expressed rapidly at high density during early MØ differentiation in vitro. Among conventional mAbs that do not react with MO we found those against the transferrin (TF)-receptor, the BA-2, and the PCA1 antigen to label MØ. MØ matured in vivo and isolated from body fluids were positive with some but not all MAX mAbs. Distinctive patterns were observed with pulmonary MØ, exudate MØ from pleural and peritoneal effusions, synovial fluids, and early lactation milk. MØ from the alveolar space, for example, constantly expressed the MAX.2 antigen but not the MAX.3 antigen. Pleural effusion MØ, however, did not react with the MAX.1 mAb, but in most cases, it did react with the MAX.3 mAb. The detection of novel differentiation antigens, all expressed on monocyte-derived MØ but differently expressed on site-specific MØ in situ, underlines the remarkable heterogeneity among human MØ. The expression of these antigens is flexible because those MAX antigens that were not expressed in situ could be induced if cells from distinct tissue sites were cultured in vitro for several days. MAX mAbs may be of potential value to study both the sequential stages of maturation within the MØ lineage as well as differential developments induced by various culture conditions in parallel to environmental factors in vivo.

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THE MONONUCLEAR PHAGOCYTE SYSTEM comprises cells of considerable heterogeneity in terms of localization, morphology, metabolism, and function.[1-3] It seems likely that there are still more members of this cell family whose delineation comes from the common bone marrow stem cell committed to monocyte-macrophage differentiation. This can be proven or disproven if suitable markers become available. Whereas cumulative knowledge exists about the development of cells from bone marrow progenitors to monocytes (MO) entering the peripheral blood, data characterizing the terminal maturation beyond the blood monocyte stage of differentiation are still limited. The anatomical site may provide the environmental signal for the immigrating MO to develop into the individual cell type, eg, bone matrix may induce osteoclast formation and other local factors may be responsible for the development of hepatic Kupffer cells, pulmonary macrophages (MØ), microglia cells, fluid phase, and tissue or granulomatous MØ. On the other hand, because remarkable heterogeneity already exists within the circulating monocyte population,[2-10] all of these mature cell types may derive from predestined monocyte subsets that are attracted to the specific anatomical site to which they are designated. The aim of this study was to detect and follow the expression of differentiation antigens on cells of the monocyte-macrophage lineage. We have used monocyte-derived MØ cultured on hydrophobic Teflon foils[11,12] as immunizing cells for the development of monoclonal antibodies (mAbs) with specificity for surface molecules on terminal in vitro differentiation stages of human MØ. Here we report on the first five mAbs of the MAX series that could be studied at sequential stages of MØ differentiation in vitro. In addition, three nonlineage-restricted antigens (TF-receptor, BA-2, and PCA1) that are not present on blood MO could be found after in vitro differentiation. It was found that in vivo-matured MØ obtained from various body fluids were of considerable diversity in terms of expression of the differentiation antigens described.

MATERIALS AND METHODS

Generation of MAX mAbs

Two-month-old BALB/c mice were injected five times intraperitoneally at weekly intervals with 5×10^6 monocyte-derived MØ that were collected from hydrophobic Teflon bags after culture of blood-borne MO for at least ten days (for characterization of the cells see Table 1). Three and a half days after the last injection, immune spleen cells were fused with the HAT-sensitive line X63Ag8.653.[14,15] Hybridoma supernatants were tested for binding to autologous B cells, T cells, MO, and monocyte-derived MØ by a cell-ELISA (enzyme-linked immunosorbent assay)[15] and by the immunoperoxidase slide technique[16] as described later. Finally, five hybridomas were selected because of their ability to discriminate between MO and MØ. They were subjected to recloning and further expansion. Cytofluorometric analysis (ORTHO 50-11) was used to confirm the reactivity pattern as evaluated by cell-ELISA and the immunoperoxidase method.[15]

MAbs to Cell Surface Antigens Used in This Study

OKT3, OKT4, OKT8, OKT6, OKT9, OKM1, and OKIa1 were purchased from Ortho Pharmaceutical Corp, Raritan, NJ; antihuman

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Table 1. Comparison of Blood MO and Monocyte-derived MØ Used as Immunizing Cells for the Development of MAX. Monoclonal Antibodies

	MO	MØ
Multinucleated cells (%)*	<1	12–18
Protein (μg/10^6 cells)	32 ± 15	376 ± 115
DNA (μg/10^6 cells)	5.7 ± 0.2	12 ± 4
Nonspecific esterase (%)	30–80	>90
Tartrate-resistant acid phosphatase (%)	<1%	>90
Peroxidase (%)	80–90	None
Surface antigens (%):		
OKM1, anti-mono, My4, MØ S39, Ki-M1 and Ki-M5	>90[2]	>90
OKIa1	60–75	>90
anti-C3b	75–90	>90
anti-IgG$_1$	>90	77 ± 8
anti-IgG$_2$, anti-IgG$_3$, and anti-IgG$_4$	None	None
Rosette formation with IgG-coated sheep erythrocytes (%)	>90	>90
Phagocytosis of IgG-coated sheep erythrocytes (%)	65–80	75–85
Intracellular ferritin (ng/10^6 cells)	<25	350–1500
Lucigenin-depdendent chemiluminescence (CL)† (mV s of 2 × 10^5 cells)	40 ± 19	125 ± 49
Phorbol ester-stimulated CL (stimulation index)	5.8 ± 2	<1.5
Zymosan-stimulated CL (stimulation index)	4.3 ± 2	3–12
24-hour secretion:		
Lysozyme (μg/10^6 cells)	1.52 ± 0.47	7.34 ± 1
Ferritin (μg/10^6 cells)	<20	68 ± 22
Interleukin 1 activity‡ (cpm per 10^5 cells)	1,500 ± 240	None
Induced by 20 μg/mL LPS	56,300 ± 4,800	1,400 ± 280
Induced by 200 U/mL gamma interferon	5,700 ± 840	270 ± 45

MO were separated by adherence for one hour on plastic dishes and then tested after overnight culture; MØ were obtained from 10- to 15-day-old MO cultures; for additional experimental details, see refs. 19–21.

*Percentage of total cells as evaluated by immunoperoxidase staining.

†Measured with a LKB Wallac 1250 Luminometer.

‡Evaluated in the mouse thymocyte proliferation assay.[13]

monocyte from Bethesda Research Lab Inc, Gaithersburg, MD; Coulter Clone B1, BA-1, BA-2, My4, and PCA1 from Coulter Immunology, Hialeah, Fla; Leu-2, Leu-3a, Leu-3b, Leu-7, and antihuman transferrin receptor from Becton Dickinson, Erembodegem, Belgium; antihuman IgG1, IgG2, IgG3, and IgG4 from Bio-Yeda Ltd, Rehovot, Israel; Ki-1, Ki-M1, and Ki-M5 from Behringwerke, Marburg, FRG; and anti-C3b from Dakopatts, Copenhagen. MØ S39[17] was a generous gift from Dr R. Winchester, Orthopedic Institute, Hospital for Joint Diseases, New York, and anti-Tac[18] from Dr T. Waldmann, NIH, Bethesda, Md. A polyclonal rabbit antitransferrin antibody was purchased from Behringwerke AG.

Cells

Blood monocytes. Buffy coat cells from normal blood donors or whole blood from patients with acute and chronic monocytic leukemias (classified by morphology, cytochemistry, and surface antigen analysis) were diluted 1:1 with phosphate-buffered saline (PBS) and centrifuged over Ficoll-Hypaque (F/H) to separate mononuclear cells (MNCs) from granulocytes and red cells. MO were separated from lymphocytes usually by adherence: 3 × 10^6 MNCs/mL RPMI 1640 (supplemented with L-glutamine, antibiotics, 5 × 10^{-5} mol/L 2-mercaptoethanol, and vitamins) plus 20% fetal calf serum (FCS) were incubated for 60 minutes in plastic Petri dishes. The nonadherent cells were removed by repeated washings with warm serum-free medium and the adherent cells (>90% MO as revealed by morphology and phenotype analysis) incubated overnight in RPMI 1640 (supplemented with MEM vitamins, penicillin 50 U/mL, streptomycin 50 μg/mL, 2-mercaptoethanol 5 × 10^{-5} mol/L, L-glutamine 2 mol/L) plus 10% human AB-group serum. MO could then be recovered from the dishes at 4 °C by vigorous pipetting. In some experiments, the MNCs were suspended in 0.1% EDTA containing PBS and loaded onto an elutriator centrifuge (J2-21, standard chamber, Beckman, Stockholm) with a flow rate of 10 mL/min. Lymphocytes were removed at 10 mL/min and 12.4 mL/min with 200 mL and 100 mL of PBS, respectively. The MO-enriched cell fraction was then collected at a flow rate of 14.0 mL/min with 150 mL PBS and contained >70% MO as judged by phenotype analysis.

Alternatively, MNCs were layered on top of a continuous Percoll (Pharmacia Fine Chemicals, Freiburg, FRG) gradient with densities ranging from 1.06 g/mL to 1.08 g/mL and centrifuged for 20 minutes at 1,000 g. MO-enriched fractions of about 70% purity could be collected from a band just below the dead cells that did not enter the gradient.

Regardless of the isolation procedure used, MO were then placed into rectangular bags of hydrophobic Teflon foils (Biofolie 25, Heraeus, Hanau, FRG) at a concentration of 3 × 10^5 cells per milliliter RPMI 1640 plus 10% human AB serum and cultured for up to 15 days.[12] Contamination with lymphocytes was confined to maximum of 8%. At different times after the initiation of the culture, MØ could be harvested from the bags and taken for phenotype analysis.

Granulocytes. After F/H centrifugation of diluted buffy coat cells, the pelleted granulocytes and red cells were resuspended in equal volumes of PBS and hydroxyethylstarch. After sedimentation for 30 minutes at 1 g the granulocyte-rich supernatant was centrifuged and the remaining erythrocytes lysed by five-minute exposure to 0.16 mol/L ammonium chloride and 0.17 mol/L trishydroxymethylaminomethan (Tris, Merck, Darmstadt, FRG) at 37 °C.

Megakaryocytes. Megakaryocytes were identified by morphology in bone marrow MNC suspensions obtained from healthy volunteers by posterior iliac crest aspiration and subjected to F/H centrifugation.

Thrombocytes. Thrombocytes were collected from the plasma supernatants after F/H centrifugation and sedimented for 10 minutes at 800 g.

T and B cells. Cells were enriched by rosetting MNCs with AET-treated sheep erythrocytes and subsequent centrifugation over F/H.[19] T cells were activated by incubation with concanavalin A (2 μg/mL) for 72 hours.

Pulmonary MØ. Macrophages were collected from the broncho-alveolar lavage fluids of healthy volunteers and patients undergoing diagnostic bronchoscopy after giving informed consent.

Pleural and peritoneal MØ. Macrophages were isolated from effusion fluids secondary to heart failure, portal hypertension, or malignancies.

Synovial MØ. Macrophages were collected from samples obtained by diagnostic puncture of knee joints.

Milk MØ. Macrophages were obtained from breast milk samples taken at day 3 to day 9 postdelivery.

Human tumor cell lines. DHL-1 (diffuse histiocytic lymphoma, from Dr M. Scott, Stanford University, Calif), U937 (histiocytic lymphoma, from Dr K. Nielsson, Wallenberg Lab, Uppsala, Sweden), HL-60 (promyelocytic leukemia, from Dr R. Vogler, Emory University, Atlanta), LBL6078 and LBL3283 (B lymphoblastoid cells), Mel-1 and Mel-2 (melanoma xenografts in nude mice, from Dr H. Fiebig, Medizinische Klinik, Freiburg, FRG), and Colo38 (melanoma cells, from Dr G. Moroni, University of Napoli, Italy).

Immunoperoxidase Slide Technique

For surface marker analysis, the cells were washed three times with cold PBS and transferred onto glass slides coated with 0.1% alcian blue in hydroxyethylstarch. After attachment of the cells to the negatively charged glass surface, they were fixed at 4 °C with 0.05% glutaraldehyde in 0.1 mol/L PBS containing 10% glucose. They were then washed with a glycine containing medium and preincubated with a gelatine containing medium to prevent immunoglobulin binding to the glass surface. The staining procedure included the following steps: (1) 15-minute incubation with mAbs diluted in gelatine containing medium; (2) five-minute incubation with rabbit antimouse Ig (Dakopatts) diluted 1:1,000 and absorbed with normal human serum and swine serum for five minutes; (3) another five-minute incubation with swine antirabbit Ig (Dakopatts) absorbed as described; (4) incubation with peroxidase-antiperoxidase complex from rabbits (Dakopatts) diluted 1:20; (5) incubation with diaminobenzidine-H_2O_2 for ten minutes, followed by postfixation with OsO_4. The slides were covered with a solution of 80% glycerine and 0.125% glutaraldehyde in 0.1 mol/L PBS. For detection of rabbit antibodies, the first two steps were omitted. The different sandwich sera served as negative controls. Unspecific Fc-receptor–mediated binding could be excluded by negative reactions with mouse IgG (MsIgG, Coulter Immunology) and several mAbs of different Ig class that did not react with the cells to be tested. Two hundred cells were evaluated for mAb reactivity and were counted as positive when dark brown staining of the membranes was seen. In a few experiments the influence of the fixation reagent on the antigenic determinants was tested by adding mAbs to the cells before the fixation step. Data are usually expressed as percentage of positive macrophages. For intracellular staining, the cells were dried on alcian blue-coated glass slides in a medium containing 0.2% bovine serum albumin. After fixation with a solution of acetone, ethanol, and formaline (80:20:0.2) on ice for five minutes, the slides were transferred to 80% acetone and then dried quickly. This was followed by hydration in acetone of decreasing concentrations from 100% to 50% and, finally, in PBS. The slides were then incubated for 15 minutes at room temperature with a gelatine containing medium with 10% swine serum, 10% human serum, and 10% glycerine, which was also used for dilution of mAbs, rabbit antimouse Ig, swine antirabbit Ig, and the peroxidase-antiperoxidase complex. The staining procedure was performed as described but each incubation was extended to 15 minutes.

RESULTS

To develop mAbs directed against lineage-restricted human MØ differentiation antigens, we used monocyte-derived MØ cultured in hydrophobic Teflon foils.[12] This method prevents the spreading of MØ on the surface, and thus allows the recovery of cells without damage. Cells cultured in this way meet all the morphologic, cytochemical, antigenic, and functional criteria for MØ ex vivo (see Table 1 for full characterization, and also refs 12, 20, 22). In this report we give detailed information on five mAbs of the MAX series that discriminate between MO and MØ. The reactivity pattern of these antibodies is described in Table 2. Two mAbs (MAX.1 and MAX.2) were reactive with membrane structures that are selectively expressed on MØ at differentiation stages beyond the blood monocyte level and not found on any other cell type studied. MAX.3 is a similar mAb, with the exception that it also reacted with platelets and megakaryocytes. MAX.11 is infrequently expressed on a minor subset of blood MO and U937 cells. MAX.1, MAX.2, and MAX.3 each precipitated membrane molecules of distinct molecular weight: MAX.1 precipitated a 64-kd protein, MAX.2 a 200-kd, and MAX.3 a 68-kd molecule (F. Emmrich and R. Andreesen, manuscript in preparation).

The expression of MAX. antigens in vitro was independent of the separation procedure used to isolate MO from other MNCs (adherence, elutriation, or density centrifugation) and was also independent of the culture substrate used (ie, grown as a monolayer on glass or as a suspension in Teflon

Table 2. Cellular Distribution of Surface Antigens Reactive With MAX. Monoclonal Antibodies

	Monoclonal Antibody Designation			
Cells	MAX.1 (IgG1/k)	MAX.2 (IgM/k)	MAX.3 (IgG1/k)	MAX.11 (IgG1/k)
Monocyte-derived MØ	++	+	++	++
Adherent monocytes (day 1)	–	–	–	var
Granulocytes	–	–	–	–
Platelets/megakaryocytes	–	–	+	–
B cells	–	–	–	–
T cells	–	–	–	–
Activated T cells	–	–	–	–
Permanent cells lines				
L-428 (Hodgkin cells)	–	–	–	–
U-937 (histiocytic lymphoma)*	–	–	–	+
DHL-1 (histiocytic lymphoma)	–	–	–	–
HL-60 (promyelocytic leukemia)	–	–	–	–
B lymphoblastoid cells†	–	–	–	–
Melanoma cells‡	–	–	–	–

Cell distribution was evaluated by immunoperoxidase staining; hybridoma supernatants were diluted 1:10. All cell lines were tested at least three times; MO, MØ (10- to 15-day-old MO cultures), B cells (nonrosetting cell population containing 55% B1$^+$ cells, MØ and less than 2% OKT3$^+$ T cells) and T cells (>90% purity) were obtained from ten donors, whereas activated T cells (about 40% Ia$^+$-blast cells), platelets, and granulocytes were from three donors. ++, more than 90% of cells reactive; +, varying reactivity ranging from 15% to 90%; var, reactivity varied among experiments from negative to up to 20% positive cells; –, no reactivity.

*Varying portions of positive cells on repeated tests with the same line (15–80%).

†LBL 6078 and LBL 3283.

‡Xenotransplanted tumorgrafts Mel-1 and Mel-2 (tested either freshly prepared from tissue specimen or after at least three serial passages in vitro) and permanent cell line Colo 38.

foils). During MØ maturation in vitro, the differentiation antigens defined by MAX. mAbs were expressed consecutively as shown in Fig 1A through D. Whereas nearly all mature MØ reacted with MAX.1 and MAX.3, only up to 70% of the cells were positive for the MAX.2 antigen. MAX.11 antigen appeared to be the first antigen to be fully expressed (after three days) and MAX.3 the latest antigen not being detectable in three-day-old MO cultures but was fully expressed by day 9.

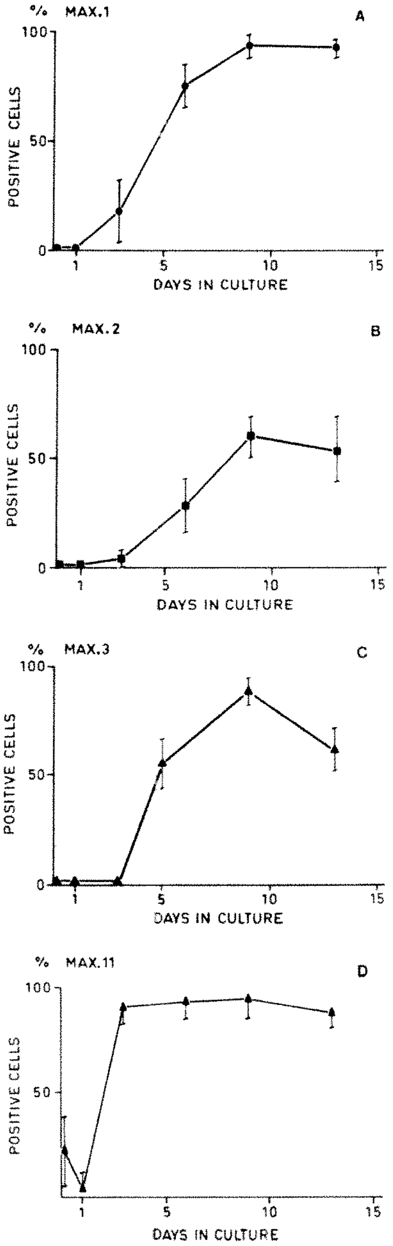

Fig 1. Sequential expression of lineage-restricted differentiation antigens during maturation in vitro. MO (4 × 10^5/mL) separated from other mononuclear cells by adherence were cultured on hydrophobic Teflon foils in supplemented RPMI 1640 plus 10% human AB group serum. At indicated days, the cells were recovered and subjected to immunoperoxidase staining with MAX.1 (A), MAX.2 (B), MAX.3 (C), MAX.11 (D). Data are expressed as percentage of positive MØ (six experiments) that were identified by their reactivity with monocyte-specific mAbs.

MAX.1 and MAX.3 antigen appeared in the cytoplasm two to three days earlier than they were expressed on the cell surface (Fig 2), whereas cytoplasmic and surface labeling of MAX.11 antigen coincided (not shown). MAX.2 antigen seemed to be sensitive to the methanol treatment that was required for intracellular staining. This treatment completely abrogated the reaction of glutaraldehyde-fixed cells with this antibody. MAX.1 and MAX.3, but not MAX.2, reactivity was lost after fixation at room temperature.

In addition, a series of conventional mAbs that do not react with blood MO was tested routinely as control for binding to in vitro-matured MØ (OKT3, OKT4, OKT6, OKT9 directed to the TF-receptor, Leu-2, Leu-3a, Leu-3b, Leu-7, B1, BA-1, BA-2, PCA1, anti-Tac, Ki-1). Interestingly, mAbs directed to the TF-receptor,[23] and the BA-2 antigen[24] labeled mature MØ to varying degrees (Fig 3). Only two days later, during in vitro differentiation, TF molecules were detected on the cell membrane (Fig 3A). The PCA1 antigen[25] also appeared to be expressed only on mature MØ with 17% to 72% of cells reacting (not shown). We observed in some experiments a weak reactivity of MØ with OKT4 and anti-Tac that, however, did not depend on the stage of maturation.

In order to investigate the expression after in vivo maturation of the differentiation antigens described, we isolated MØ from various body cavities (Table 3).

A considerable heterogeneity was found concerning membrane antigen expression in MØ from different sites and also between different individuals. Some generalizations seem to be possible: MAX.3 was absent from pulmonary and breast milk MØ but present on most pleural and some peritoneal MØ. MAX.1 could be detected only on minor portions of pulmonary and peritoneal MØ but never on pleural or breast milk MØ. However, if pulmonary and peritoneal MØ with distinct surface phenotypes were cultured on hydrophobic Teflon foils, the cells readily expressed these MAX antigens that were missing in situ (Table 4).

Up to now no significant difference could be detected in the MAX antigen expression on pulmonary MØ from smokers and nonsmokers, whereas with pleural and peritoneal

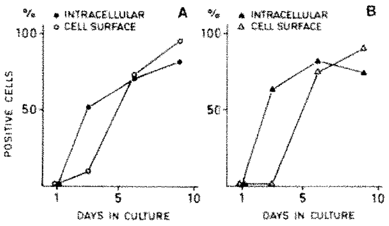

Fig 2. Intracytoplasmic detection of two MAX. differentiation antigens as compared with cell surface expression. (A) MAX.1 and (B) MAX.2. As described in Fig 1, MO were isolated and cultured on hydrophobic Teflon foils. Immunoperoxidase staining was performed on either glutaraldehyde-fixed cells (cell surface labeling) or cells that were dried on alcian blue-coated slides before fixation with acetone (intracellular staining). For experimental details, see Materials and Methods. Data are from one representative experiment.

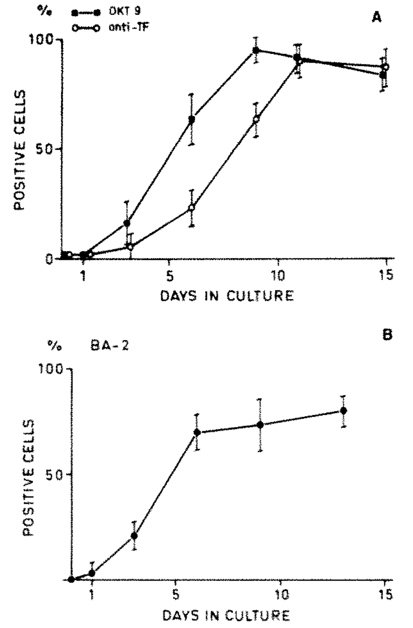

Fig 3. Expression of nonlineage-restricted differentiation antigens during macrophage maturation in vitro: the TF-receptor and surface TF (A) and the BA-2 antigen (B). For experimental details, see Fig 1.

MØ, a great variability among patients in terms of MAX antigen expression was seen. This is possibly due to the different diseases that had led to pleural or peritoneal effusions, a problem that will be studied in more detail. No reactivity of MAX.1, MAX.2, and MAX.3 was found with monocytic leukemias, which usually expressed the MAX.11 antigen. Preliminary experiments indicate that human Kupffer cells elutriated from cell suspensions obtained by perfusion of liver with collagenase do not express MAX.1, MAX.2, and MAX.3, but could be induced in culture to express MAX.2 (R. Andreesen and H. G. Leser, to be published).

Studies concerning in situ localization of MAX differentiation antigens using a sensitive four-layer immunoperoxidase technique are in progress.

DISCUSSION

We have analyzed the expression of antigens on human mononuclear phagocytes as they undergo terminal maturation. Differentiation from monocyte precursor cells as a more or less homogenous population in the peripheral blood leads to heterogeneic mature cells residing in various anatomical sites of the organism. Despite the changes in morphology, biochemistry, and function that occur while MO transform into mature cells, surprisingly little is known about the loss or acquisition of surface antigens beyond the blood monocyte stage of differentiation. With the new mAbs, we have identified two molecules (MAX.1 and MAX.2 antigens) on mature MO which are absent from MO and any other cell type tested so far, and a third molecule (MAX.3 antigen), which is shared only by platelets and megakaryocytes. Other antigens have been reported that are shared by the megakaryocyte and MØ lineages, such as the MPA antigen,[26] which differs, however, from MAX.3 as it is present on freshly harvested blood MO. As has been described for immunoglobulins during B cell differentiation,[27] these maturation-related molecules are first detected in the cell cytoplasm before they appear on the outer cell surface.

All three antigens seem to be unique and different from any other reported so far: the PAM1 antigen detected by Biondi et al.,[28] like MAX.2, also binds to a 200-kd structure that, according to their studies, is not expressed on monocyte-derived MØ and is restricted to pulmonary MØ; the BMM1 mAb reported by the same authors to be specific for

Table 3. Heterogeneity of Human Macrophages as Defined by Surface Antigen Analysis

	Monoclonal Antibody Reactivity (%)				
Source of Macrophages	MAX.1	MAX.2	MAX.3	MAX.11	OKT9
Normal blood (n = 6)	None	None	None	21 ± 17 (0-48)	None
Monocytic leukemia (n = 5)	None	None	None	57 ± 28 (2-85)	<1
Monocyte-derived (Teflon bag, n = 6)	>90	61 ± 10	>90	>90	>90
Pleural effusion (n = 8)	<1	23 ± 21 (2-61)	51 ± 28 (15-80)	41 ± 27 (10-80)	3 ± 2 (0-10)
Ascites fluid (n = 12)	17 ± 18 (0-50)	16 ± 18 (0-50)	60 ± 24 (18-95)	46 ± 28 (10-90)	22 ± 16 (0-45)
Alveolar lavage fluid (n = 14)	7 ± 6 (0-16)	84 ± 8	<1	62 ± 30 (10-94)	>90
Breast milk (n = 4)	<1	7 ± 7 (0-20)	<1	39 ± 13	78 ± 10
Synovial fluid (n = 4)	7 ± 7 (0-16)	8 ± 4 (0-10)	23 ± 7	54 ± 19	11 ± 4

Monoclonal antibody reactivity was evaluated by immunoperoxidase staining on alcian blue-coated slides; MAX. mAbs were diluted 1:10 (hybridoma supernatant), OKT9 1:100 (ascites fluid). Values are given as mean percentage of positive monocyte-macrophages identified by morphology and in comparison by binding of monocyte-specific mAbs. The range within individual values is given in parentheses. MO were assayed for reactivity with mAbs in freshly prepared mononuclear cell suspensions where they represented 12% to 34% of total cells. All leukemic MO populations tested were of more than 90% purity. Except for alveolar lavage fluids, which always contained more than 80% of MØ, the MØ content in other body fluids varied from 18% to 68%.

Table 4. Induction of MAX. Differentiation Antigens on Cultured Pulmonary and Peritoneal Macrophages

	MAX Monoclonal Antibodies		
Macrophages	MAX.1 %	MAX.2 %	MAX.3 %
Pulmonary MØ (n = 5)			
Freshly isolated	4 ± 3	68 ± 14	<1
Cultured on Teflon	85 ± 8	75 ± 7	59 ± 7
Peritoneal MØ (n = 3)			
Freshly isolated	<1	<1	75 ± 12
Cultured on Teflon	95 ± 4	66 ± 8	61 ± 12

MØ were obtained from alveolar lavage fluids from patients undergoing diagnostic bronchoscopy and from ascites secondary to portal hypertension or malignancies. Reactivity as evaluated by immunoperoxidase staining is expressed as the mean percentage of total MØ count. MØ were cultured at 3×10^5/mL in RPMI 1640 plus 10% AB serum for six days.

breast milk MØ in fact reacted with minor subpopulation of a T cell line (HPB-ALL) and a minor portion of blood MO[28]; the 27F9 antigen reported by Zwadlo et al[29] is present on melanoma cells, differs in the molecular weight, is expressed only up to 70% on monocyte-derived MØ, and can be detected on Kupffer cells. MAX.11 reacts with a lineage-restricted molecule that, however, is found to variable degrees on a minor portion of blood MO and U937 cells but is rapidly expressed on more than 95% of MØ at day 3 in Teflon cultures of MO. Somewhat similar seems to be the Mo3 antigen[30] that is found, although at low density, on the majority of blood MO and shows increased expression after overnight incubation.[30]

Other groups have developed mAbs specific for the monocyte-macrophage lineage but that are not able to distinguish between MO and mature MØ: MØ P15 and MØ S39 by Dimitriu-Bona et al,[17] Mo2 by Todd and Schlossmann,[30] some of the FMC series by Hancock et al,[31] the PHM3 by Becker et al,[32] the 3C10 and 1D9 by Van Voorhis et al,[33] and the KiM1 by Radzun and Parwaresch.[34]

The MØ antigens described here do not seem to be the result of culture artifacts as (1) they all could be detected, although in a distinctive pattern, on in vivo-matured MØ obtained from various anatomical sites; (2) non-MØ cells and cell lines were not induced to express the antigens under the same culture conditions (T cells, both activated and nonactivated, B cells, melanoma xenografts, B lines); and (3) MAX.1- and MAX.3-negative Kupffer cells do not express these antigens after comparable culture periods. These antigens are obviously not differentiation antigens for the following reasons: (1) Cell activation by endotoxin or interferon-gamma usually is completed in less than 24 hours and is reversible, whereas cell differentiation to our belief is a continuous and nonreversible process a cell is committed to undergo. (2) The kinetics of activation and differentiation are quite distinct, as MO had to be cultured three days to see expression of differentiation antigens that, in the case of MAX.2, affects only a certain part of the cells (Fig 1). (3) In fact, when MO were activated for tumor cytotoxicity by interferon gamma (up to 200 U/mL), MAX antigen expression was inhibited (to be published). (4) It has been reported that MØ maturation is accompanied by an increase in some cell activities (see Table 1 and refs 12, 20, 21, 35; however, others, like the secretion of interleukin 1 and prostaglandin E_2, are even lost at a culture stage where MAX antigens are fully expressed (Table 1, to be published in detail).

Antigenic heterogeneity of mature MØ has been shown in the rat[36,37] and, more recently, on human cells.[28] Thus, end-stage maturation in vivo does not necessarily lead to the expression of all MØ differentiation antigens; some may in fact be suppressed by environmental factors in the specific tissue site MO have migrated to. Yet, the difference in the expression of antigens all present on monocyte-derived MØ among mature MØ from various anatomical sites is of considerable interest: it may indicate a functional correlation of specific membrane structures to the properties of cells residing in a given anatomical site. As these site-specific MØ can, however, be induced to express the missing antigens on in vitro culture, it supports the hypothesis that local factors in the microenvironment control the distinct phenotypes observed. This seems to be more likely than the existence of MO subsets, which are committed to terminal differentiation into pulmonary, pleural, or peritoneal exudate MØ, respectively.

The detection of antigens unique for late in vitro differentiation stages of MØ and at the same time differentially expressed on subtypes of mature MØ of various anatomical sites might be of functional significance, as both maturation in vitro as well as generation of site-specific heterogeneity in vivo seems to be accompanied by quantitative changes in certain MØ functions: (a) the capacity to kill tumor cells in vitro,[21,35,38] (b) the bactericidal activity[39] and hydrogen peroxide metabolism,[40] (c) the accessory cell function[41] and production of interleukin 1,[42,43] and our own unpublished results).

MØ differentiation in vitro is followed by an increase in ferritin synthesis,[21] a change in the glycolipid pattern (M. Rössle and R. Andreesen, submitted for publication), and in cell adhesion behavior,[44] the induction of cAMP-dependent protein kinase 1[45] and calmodulin-binding protein,[46] and the expression of ecto-5'nucleotidase[47] and creatine kinase.[48] Thus, it has to be established whether the expression of the MAX. antigens is related to some of those features. This is subject to current investigations. Preliminary data seem to indicate that MAX. antigens are not involved in tumor cytotoxicity or in oxidative metabolism and Fc-receptor-mediated phagocytosis (to be published).

Besides lineage-restricted differentiation antigens, we found also nonlineage-restricted antigens that are newly expressed (TF-receptor, BA-2, TF-R, and PCA1). The TF-receptors may serve to deliver iron to intracellular stores. The detection of cell surface TF two days later than the expression of TF receptor in culture has to be explained. It may be due to incomplete expression of TF-receptors not yet capable of binding TF. Alternatively, membrane-bound TF by itself might be a MØ product that is detected independent of the TF-receptor. Data from biosynthetic labeling experiments indicate that TF is synthesized by mature MØ and probably processed to surface membrane sites distinct from the TF-receptor reactive with the OKT9 mAb (R. Andreesen and G.

Bauer, manuscript in preparation). Both the TF-receptor and surface TF could function to facilitate tumor cell–MØ interaction, as has been shown for natural killer cell activity.[49]

Together with the MAX series, mAbs to the TF receptor, the BA-2, and the PCA-1 antigen seem to be useful for a phenotypic followup of MØ terminal maturation. The novel differentiation antigens described here may also serve to indicate distinct pathways of differentiation, although their functional significance has yet to be established.

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REFERENCES

1. van Furth R: Current view on the mononuclear phagocyte system. Immunobiology 161:178, 1982

2. Raff HV, Picker LJ, Stobo JD: Macrophage heterogeneity in man. J Exp Med 152:581, 1980

3. Forster O, Landy M (eds): Heterogeneity of Mononuclear Phagocytes. Orlando, Fla, Academic Press, 1981

4. Nunez G, Giles RC, Ball EJ, Hurley CK, Capra JD, Statny P: Expression of HLA-DR, MB, MT and SB antigens on human mononuclear cells: Identification of two phenotypically distinct monocyte populations. J Immunol 133:1300, 1984

5. Bursuker I, Goldman R: On the origin of macrophage heterogeneity: A hypothesis. RES 33:207, 1983

6. Arenson EB, Epstein MP, Seeger RC: Volumetric and functional heterogeneity of human monocytes. J Clin Invest 65:613, 1980

7. Norris DA, Morris RM, Sanderson RJ, Kohler PJ: Isolation of functional subsets of human peripheral blood monocytes. J Immunol 123:166, 1979

8. Picker LJ, Raff HV, Goldyne ME, Stobo JD: Metabolic heterogenity among human monocytes and its modulation by PGE_2. J Immunol 124:2557, 1979

9. Shen HH, Talle MA, Goldstein G, Chess L: Functional subsets of human monocytes defined by monoclonal antibodies: A distinct subset of monocytes contains the cells capable of inducing the autologous mixed lymphocytes culture. J Immunol 130:698, 1983

10. Zembala M, Uracz W, Ruggiero I, Mytar B, Pryjma J: Isolation and functional characterization of FcR^+ and FcR^- human monocyte subsets. J Immunol 133:1293, 1984

11. Munder PG, Modolell M, Wallach DFH: Cell propagation on polymeric fluorocarbon as a mean to regulate pericellular pH and PO2 in cultured monolayers. FEBS lett 15:191, 1971

12. Andreesen R, Picht J, Löhr GW: Primary cultures of human blood-borne macrophages grown on hydrophobic teflon membranes. J Immunol Methods 56:295, 1983

13. Blyden G, Handschumacher RE: Purification and properties of human lymphocyte activating factor (LAF). J Immunol 118:1631, 1977

14. Köhler G, Milstein C: Continous culture of fused cells secreting antibody of predefined specificity. Nature 256:495, 1975

15. Emmrich F, Andreesen R: Monoclonal antibodies against differentiation antigens on human macrophages. Immunol Lett 9:321, 1985

16. Bross KJ, Pangalis GA, Staatz CG, Blume KG: Demonstration of cell surface antigens and their antibodies by the peroxidase-antiperoxidase method. Transplantation 25:331, 1978

17. Dimitriu-Bona A, Burmester GR, Waters SJ, Winchester RJ: Human mononuclear phagocyte differentiation antigens. I. Patterns of antigenic expression on the surface of human monocytes and macrophages defined by monoclonal antibodies. J Immunol 130:145, 1983

18. Uchiyama T, Broder S, Waldmann TA: A Monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. J Immunol 126:1393, 1981

19. Garotta G, Neri TM: HLA-DR typing by complement-dependent B lymphocyte lysis, in Lefkovits I, Pernis B (eds): Immunological Methods, vol 2. Orlando, Fla, Academic Press, 1981, p 163

20. Andreesen R, Osterholz J, Bross KJ, Schulz A, Luckenbach GA, Löhr GW: Cytotoxic effector cell function at different stages of human monocyte-macrophage maturation. Cancer Res 43:5931, 1983

21. Andreesen R, Osterholz J, Bodemann HH, Bross KJ, Costabel U, Löhr GW: Expression of transferrin receptors and intracellular ferritin during terminal differentiation of human monocytes. Blut 49:195, 1984

22. Andreesen R, Osterholz J, Luckenbach GA, Costabel U, Schulz A, Speth V, Munder PH, Löhr GW: Tumor cytotoxicity of human macrophages after incubation with synthetic analogues of 2-lysophosphatidylcholine. JNCI 72:53, 1984

23. Trowbridge IS, Omary MB: Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. Proc Natl Acad Sci USA 78:3039, 1981

24. Kersey JH, LeBien TW, Abramson CS, Neuman R, Sutherland R, Greaves M: p 24: A human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. J Exp Med 153:726, 1981

25. Anderson KC, Park EK, Bates MP, Leonard RCF, Hardy R, Schlossman SF, Nadler LM: Antigens on human plasma cells identified by monoclonal antibodies. J Immunol 130:1132, 1983

26. Burckhardt JJ, Anderson WHK, Kearney JF, Cooper MD: Human blood monocytes and platelets share a cell surface component. Blood 60:767, 1982

27. Raff MC, Megson M, Owen JJT, Cooper MD: Early production of intracellular IgM by B-lymphocyte precursors in mouse. Nature 259:224, 1976

28. Biondi A, Rossing TH, Bennett J, Todd RE: Surface membrane heterogeneity among human mononuclear phagocytes. J Immunol 132:1237, 1984

29. Zwadlo G, Bröcker EB, v. Bassewitz DB, Feige U, Sorg C: A monoclonal antibody to a differentiation antigen present on mature human macrophages and absent from monocytes. J Immunol 134:1487, 1985

30. Todd RF III, Schlossman SF: Analysis of antigenic determinants on human monocytes and macrophages. Blood 59:775, 1982

31. Hancock WW, Zola H, Atkins RC: Antigenic heterogeneity of human mononuclear phagocytes: Immunohistological analysis using monoclonal antibodies. Blood 62:1271, 1983

32. Becker GJ, Hancock WW, Kraft N, Lanyon HC, Aktins RC: Monoclonal antibodies to human macrophage and leukocyte common antigens. Pathology 13:669, 1981

33. Van Voorhis WC, Steinman RM, Hair LS, Luban J, Witmer MD, Koide S, Cohn ZA: Specific antimononuclear phagocyte monoclonal antibodies. J Exp Med 158:126, 1983

34. Radzun HJ, Parwaresch MR: Differential immunohistochemical resolution of the human mononuclear phagocyte system. Cell Immunol 82:174, 1983

35. Rinehardt JJ, Gormus BJ, Lange P, Kaplan ME: Characterization and comparison of human monocyte and macrophage-induced mmmmmmmmm

36. Dijkstra CD, Döpp EA, Joling P, Kraal G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. Immunology 54:589, 1985

37. Rumpold H, Förster O, Böck G, Swetly P, Riedl M: Antigenic heterogeneity of rat macrophages. A monoclonal antibody reacting only with alveolar but not with other types of macrophages. Immunology 45:637, 1982

38. Bordignon D, Avallone R, Peri G, Polentarutti N, Mangioni C, Mantovani A: Cytotoxicity on tumor cells of human mononuclear phagocytes: Defective tumoricidal capacity of alveolar macrophages. Clin Exp Immunol 41:336, 1980

39. McLeod R, Bensch KG, Smith SM, Remington JS: Effects of human peripheral blood monocytes, monocyte-derived macrophages, and spleen mononuclear phagocytes on *Toxoplasma gondii.* Cell Immunol 54:330, 1980

40. Nakagawara A, Nathan CF, Cohn ZA: Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. J Clin Invest 68:1243, 1981

41. Mayernik DG, Ul-Haq A, Rinehart JJ: Differentiation-associated alteration in human monocyte-macrophage accessory cell function. J Immunol 130:2156, 1983

42. D'Onofrio C, Lohmann-Matthes ML: Interleukin-1 and PGE_2 synthesis in monocyte derived human macrophages during in vitro maturation. Immunbiology 165:255, 1983

43. Mayernik DG, Haq A, Rinehart JJ: Interleukin-1 secretion by human monocytes and macrophages. J Leucocyte Biol 36:551, 1984

43a. Rössle M, Haag K, Gerok W, Andreesen R: Characterization of gangliosides and neutral glycosphingolipids in human monocyte-macrophage differentiation. J Leukocyte Biol (submitted for publication)

44. Giavazzi R, Hart IR: Mononuclear phagocyte adherence in the presence of laminin. Exp Cell Res 146:391, 1983

45. Wenger GD, O'Dorisio MS: Induction of cAMP-dependent protein kinase I during human monocyte differentiation. J Immunol 134:1836, 1985

46. Orlow SJ, Rosenstreich DL, Pifko-Hirst S, Rosen OM: Purification and distribution of a novel macrophage-specific calmodulin-binding glycoprotein. J Immunol 134:449, 1985

47. Edelson PJ: Plasma membrane ectoenzymes: Macrophage differentiation markers, in Forster O, Landy M (eds): Heterogeneity of Mononuclear Phagocytes. Orlando, Fla, Academic Press, 1981, p 143

48. Loike JD, Kozler VF, Silverstein SC: Creatine kinase expression and creatine phosphate accumulation are developmentally regulated during differentiation of mouse and human monocytes. J Exp Med 159:746, 1984

49. MacDougall SL, Sullivan AK: Membrane-associated transferrin-like molecules on human natural killer cells: A potential ligand for interaction with target cells. Immunobiology 167:189, 1984

Inhibition of Neutrophil Cytolysin Production by Target Cells

By Franco Dallegri, Franco Patrone, Alberto Ballestrero, Guido Frumento, and Carlo Sacchetti

Neutrophils, triggered by heat-aggregated human IgG (Agg.IgG), were found to lyse chicken red blood cells (CRBC) as determined by a ^{51}Cr release method. The lysis was inhibited by azide, catalase, chloride-free medium and amino acids, suggesting the requirement for myeloperoxidase (MPO), hydrogen peroxide (H_2O_2), chloride ions (Cl^-), and hypochlorous acid (HOC1), respectively. These results indicate that neutrophils lyse CRBC through an HOCl-(ie, MPO-H_2O_2-Cl^-) dependent process. Although HOCl can react with neutrophil-derived nitrogenous (*N*–) compounds to yield chloramines, the main and well-characterized chloramines did not play a direct role in the lysis of CRBC in our model system. Thus, it appears that lysis is due either to HOCl or to an unknown compound derived from and with characteristics similar to HOCl. When CRBC were replaced with HRBC targets, no lysis could be observed. Treatment of HRBC with carmustine, to inhibit the glutathione cycle, did not affect the cell resistance to lysis by neutrophils. Conversely, the inhibition of HRBC catalase activity with aminotriazole (AT) made the cells susceptible to neutrophil-mediated HOCl-dependent lysis: this suggests that HRBC escape lysis by neutrophils through an AT-inhibitable, ie catalase-dependent, process. Through an identical catalase-dependent process, HRBC were capable of efficiently preventing the H_2O_2 and HOCl recovery from Agg.IgG-triggered neutrophils, tested under experimental conditions similar to those used for cytolytic assays. Together, these data suggest that HRBC targets, endowed with high catalase activity, escape neutrophil-mediated lysis by consuming (by catalase) neutrophil-derived H_2O_2, so that HOCl cannot be produced in amounts sufficient to promote lysis. Parallel experiments, performed with AT-treated CRBC, showed that these cells, endowed with a relatively low catalase content, only partially limit neutrophil cytolytic efficiency by a process qualitatively similar to that observed with HRBC targets. The results provide evidence that target cells can restrain neutrophil cytolytic efficiency by interfering with the MPO-H_2O_2-Cl system through their catalase activity.

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THE INJURY OF TISSUE CELLS by activated neutrophils is generally believed to play a central role in the pathogenesis of immune complex-induced tissue damage.[1,2] Most of our understanding of the mechanisms of such neutrophil-mediated injury has come about with the development of experimental models, in which neutrophils were activated by a soluble (phorbol myristate acetate) or particulate (opsonized zymosan) stimulus in the presence of eukaryotic target cells (as reviewed in ref 3). In addition, we have recently used heat-aggregated human IgG (fixed to micropore filters) as an activating agent,[4] to evoke in neutrophils an array of responses that better mimic the biological function of these cells in immune complex-dependent inflammatory process.[5] In each of these systems, activated neutrophils nonspecifically lyse surrounding bystander target cells by using oxidative means.[3,4,6] At the present status of our knowledge, such type of lysis is widely assumed to reflect the capacity of the neutrophils to overcome the ability of the target cells to detoxify oxygen-derived cytolysin(s).[3,7] In the present paper, by the use of Agg.IgG-triggered neutrophils tested against chicken (C) and human (H) red blood cells (RBC), we provide evidence that target cells can efficiently interfere with an early step of the neutrophil cytolytic pathway. This target cell capacity may represent an additional mechanism whereby tissue cells, surrounding activated neutrophils try to maintain their biological integrity.

MATERIALS AND METHODS

Neutrophils. Heparinized venous blood (heparin 10 U/mL, Liquemin, Roche, Milan) was obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as previously described.[4] Contaminating erythrocytes were removed by hypotonic lysis.[4] The neutrophils were washed three times with Hanks' balanced saline solution lacking phenol red (HBSS, Flow Lab, Ltd, Irvine, Scotland) and resuspended in HBSS supplemented with 0.1 mg/mL bovine serum albumin (BSA, Calbiochem-Behring, San Diego) (BSA–HBSS). Final cell suspensions contained 97% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide–fluorescein diacetate test.[8]

Target cells. Human (H) and chicken (C) red blood cells (RBC) were washed four times with HBSS and labeled (60 minutes, 37 °C) with 100 to 200 μCi of $Na_2(^{51}Cr)O_4$ (Radiochemical Centre, Amersham, England), as previously described.[4] The cells were then washed three times with HBSS and resuspended in BSA-HBSS. When required, labeled CRBC or HRBC (8×10^6/mL in BSA–HBSS) were divided into three aliquots and respectively incubated (60 minutes, 37 °C) with 20 mmol/L 3-amino-1,2,4-triazole (AT, Sigma Chemical Co, St Louis) plus 2 mmol/L L-ascorbic acid (Sigma) (RBC^{AT})[9] or with 2 mmol/L L-ascorbic acid or with BSA–HBSS alone. Similarly, when required, labeled CRBC or HRBC (8×10^6/mL in BSA–HBSS) were divided into three aliquots and respectively incubated (60 minutes, 37 °C) with 100 μg/mL carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU, Sintesa S.A., Brussels] dissolved in ethanol (final ethanol concentration, 0.1%) (RBC^{BCNU}),[7] or with 0.1% ethanol, or with BSA–HBSS alone. When required, RBC (2×10^7/mL) were incubated (30 minutes, 37 °C) with 0.1 mmol/L 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS, Sigma) to inhibit the anion-transport system (RBC^{DIDS}).[10,11] After incubation, the cells were washed three times with HBSS and resuspended in BSA–HBSS.

Aggregated IgG. Human IgG Cohn fraction II (Globuman Berna, Istituto Sieroterapico Berna, Como, Italy) were aggregated by heating at 63 °C for one hour.[4] The aggregated IgG (Agg.IgG) were subdivided in aliquots and stored at −20 °C. The same batch of Agg.IgG was used throughout.

Cytolytic assays. Neutrophil-mediated RBC lysis was studied

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by a ^{51}Cr release method, as previously described.[4] Briefly, 10^5 ^{51}Cr-labeled RBC were mixed with neutrophils at appropriate concentrations. Incubations were initiated by the addition of Agg.IgG to reach the final concentration of 0.2 mg/mL, followed by centrifugation of the samples at 50 g (five minutes, 22 °C). Tests were performed in triplicate and in a final volume of 0.2 mL, using round-bottom microplates (Sterilin, Teddington, England). After incubation for four hours at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, the ^{51}Cr release was determined in the cell-free supernatants. The percentage of lysis (percentage of ^{51}Cr release) was calculated according to the formula (E-S)/(T-S) × 100, where E is the cpm released in the presence of effector cells, T is the cpm released after lysing target cells with 5% Triton X-100 (Sigma), S is the cpm spontaneously released by target cells in the absence of effector cells (in each case $\leq 10\%$).

In order to determine the lytic capacity of different compounds (hypochlorous acid, HOCl; monochloramine, NH_2Cl; taurine mono- and di-chloramine, TauNHCl and $TauNCl_2$), aliquots of these compounds were added to 10^5 ^{51}Cr-labeled RBC. Tests were performed in a final volume of 0.2 mL, using round-bottom microplates (Sterilin). After incubation (four hours, 37 °C), without agitation, the samples were centrifuged (five minutes, 100 g) and the percentage of ^{51}Cr release was determined in the cell-free supernatants.

Superoxide release. Release of superoxide (O_2^-) by Agg.IgG-triggered neutrophils was studied in triplicate by using a modification of the method of Babior et al,[12] as previously described.[13]

Hydrogen peroxide release. Hydrogen peroxide (H_2O_2) release was studied by the homovanillic acid (HVA)-horseradish peroxidase (HRP) method of Ruch et al.[14] Tests were performed in triplicate under experimental conditions similar to those used for cytolytic assays, except that incubations were done in Falcon plastic tubes (12 × 75 mm, Falcon Plastics, Oxnard, Calif). Briefly, 5 × 10^5 neutrophils were incubated (37 °C) with and without 0.2 mg/mL Agg.IgG in the presence of 200 μM HVA (Sigma) plus 2 U/mL HRP (Sigma). When required, an appropriate number of RBC targets was added. The cells were sedimented by centrifugation (50 g, five minutes) at the beginning of the assay. The reactions, performed in a final volume of 2 mL, were stopped after 30 minutes with 0.25 mL of 0.1 mol/L glycine-NaOH buffer (pH 12), containing 25 mmol/L EDTA. The amount of H_2O_2 in the cell-free supernatants was determined fluorimetrically (λ_{ex} 312 nm, λ_{em} 420 nm) using appropriate standards of H_2O_2. Preliminary experiments showed that the amount of the H_2O_2 recovered from Agg.IgG-triggered neutrophils incubated (30 minutes) as a pellet equals that recovered from Agg.IgG-triggered neutrophils incubated as a pellet during the first five minutes of the assay period (30 minutes) and then resuspended and maintained in suspension (the H_2O_2 recovery from pelleted neutrophils reached the plateau after 40 minutes of incubation, while the recovery from pelleted and then resuspended neutrophils increased also after 40 minutes of incubation to reach the plateau after ~120 minutes).

Hypochlorous acid production. The generation of hypochlorous acid (HOCl) by Agg.IgG-triggered neutrophils was quantitated by the taurine trapping technique, as described by Weiss et al.[15] The conditions of the assay were identical to those used for H_2O_2 release, except that reactions were carried out in a final volume of 1 mL. Briefly, 5 × 10^5 neutrophils were incubated (30 minutes, 37 °C) with 0.2 mg/mL Agg.IgG in the presence of 20 mmol/L taurine (Sigma). Tests were performed in the presence and in the absence of RBC targets. The cells were sedimented by centrifugation (50 g, five minutes) at the beginning of the assay. At the end of the incubation period, catalase (600 units, Sigma) was added to reduce any residual H_2O_2. The samples were then incubated (22 °C, five minutes) with 100 nmol/L of 5-thio-2-nitrobenzoic acid (Nbs), prepared by reducing 5-5′-dithiobis (2-nitrobenzoic acid) (Nbs_2, Sigma) as described by Aune and Thomas.[16] The amounts of Nbs oxidized were determined by measuring the absorbance at 412 nm of the cell-free supernatants ($\epsilon = 1.36 \times 10^4$ mol/L^{-1} cm^{-1}). The quantities of HOCl trapped by taurine (yielding taurine mono-chloramine, TauNHCl) were calculated, taking into account that 1 mol TauNHCl oxidizes 2 mol of Nbs.[15] As for the H_2O_2 assay, the method of HOCl detection maintained its efficiency even when tests were carried out with cells incubated as a pellet for 30 minutes. Under the conditions described, the uptake of TauNHCl by RBC, by the DIDS-inhibitable anion-transport system,[11] did not affect the TauNHCl recovery from neutrophils, incubated with RBC and taurine (the amounts of TauNHCl recovered in the presence of RBC equalled those recovered in the presence of RBC^{DIDS}).

Enzyme assays. Total neutrophil content of myeloperoxidase (MPO) was measured after lysing the cells by sonication, followed by freezing-thawing and solubilization with 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma).[17] The MPO activity released by neutrophils, incubated (1 hour, 37 °C) with 0.2 mg/mL Agg.IgG, was determined in HTAB untreated cell-free supernatants. The MPO assay was done by using 0.167 mg/mL *O*-dianisidine (Sigma) and 0.1 mmol/L H_2O_2 in 50 mmol/L phosphate buffer, pH 6. One unit of enzyme activity was defined as that oxidizing 1 μmol of *O*-dianisidine/min/25 °C (OD 550, $\epsilon = 11.3$ mmol/L^{-1} cm^{-1}). For determination of enzyme activities, RBC targets were lysed as described by Beutler[18]: catalase activity was determined by recording the rate of decomposition of H_2O_2 at 230 nm ($\epsilon = 0.071$ mmol/L^{-1} cm^{-1});[18] glutathione (GSSG) reductase (R) activity was determined by following the GSSG-dependent oxidation of NADPH at 340 nm ($\epsilon = 6.22$ mmol/L^{-1} cm^{-1})[18]; glutathione (GSH) peroxidase (P) activity was determined by measuring the *t*-butyl hydroperoxide-dependent rate of formation of GSSG from GSH by means of the GSSG-R reaction.[18] Preliminary experiments showed that these RBC enzyme activities were not affected by the ^{51}Cr-labeling procedure.

Special materials. Superoxide dismutase (SOD, type I, bovine blood, EC 1.15.1.1, Sigma), stored at −20 °C at a concentration of 5 mg/mL in distilled water, was diluted in medium before use. Catalase (bovine liver, EC 1.11.1.6), taurine, L-alanine, L-serine, mannitol, and benzoate were purchased from Sigma. Ethanol, sodium azide (NaN_3), and $(NH_4)_2SO_4$ were from Merck, Darmstadt, FRG. Hypochlorous acid (HOCl) was generated by adding sodium hypochlorite (NaOCl, B.D.H. Ltd, Poole, UK) into solutions buffered at pH 7.4, taking into account that 50% HOCl is ionized to OCl^- at this pH (OD 292, $\epsilon = 350$ mol/L^{-1} cm^{-1} for OCl^-).[19] Taurine monochloramine (TauNHCl) and taurine dichloramine ($TauNCl_2$) were synthesized from taurine and HOCl, as described by Thomas et al[20]; monochloramine (NH_2Cl) was synthesized from NH_4^+ and HOCl, as described by Grisham et al.[21] Other reagent-grade compounds were used as obtained from commercial suppliers. In some experiments, as it will be stated, BSA-HBSS medium was replaced with a Cl^--free 0.1 mol/L phosphate buffer, pH 7.4, with 1.5 mmol/L $MgSO_4$,[22] supplemented with 1 mg/mL glucose (Merck) and 0.1 mg/mL BSA (Calbiochem-Behring) (Cl^--free medium).

All data are expressed as the mean ±1 SEM of the indicated number (n) of experiments, unless otherwise stated.

RESULTS

Neutrophils were capable of lysing CRBC in the presence of Agg.IgG (percentage ^{51}Cr release: 52.1 ± 2.0, n = 10, effector [E]-target [T] = 1:1), but not in the presence of native IgG (percentage of ^{51}Cr release: −0.2 ± 0.4, n = 4, E:T = 1:1). Binding or ingestion of CRBC by neutrophils was never observed.

To clarify the role of oxygen metabolites in our cytolytic system, CRBC lysis by Agg.IgG-triggered neutrophils was studied in the presence of SOD to remove the generated O_2^- (29.1 ± 1.7 nmol/10^6 cells/30 min, n = 3) and in the presence of catalase to degrade H_2O_2 (H_2O_2 release: 17.9 ± 2.3 nmol/10^6 cells per 30 minutes, n = 3). As shown in Table 1, CRBC lysis was unaffected by SOD and prevented by catalase (catalase: 4,000 U/mL = 100 μg/mL; the lysis was unaffected by adding either 100 μg/mL heat-inactivated catalase or 2.5 mg/mL BSA). Therefore, lysis requires H_2O_2 but not O_2^-. This conclusion excludes the involvement of hydroxyl (· OH) radicals, thought to be made by a metal-catalyzed reaction between O_2^- and H_2O_2.[23] In fact, benzoate, ethanol, and mannitol, capable of rapidly reacting with ·OH radicals,[24] had no inhibitory effect (Table 1). Although H_2O_2 might be directly involved in the lytic event, it may also act as a component of the HOCl-generating system (MPO-H_2O_2-Cl^-).[15,20,25,26] Three series of experiments suggest that CRBC lysis strictly depends on this system. First, azide (1 mmol/L) inhibited both neutrophil MPO activity and CRBC lysis (Table 1) by ~80%, suggesting the requirement for MPO in the lytic process (neutrophil MPO content = 155.9 ± 8.4 mU/10^6 cells; Agg.IgG-triggered MPO release = 3.7 ± 0.4 mU/10^6 cells per one hour; n = 3). Second, no CRBC lysis was obtained in the absence of Cl^-, also when the experiments were performed at an E:T ratio of 2:1 (instead of 1:1) in order to provide adequate amounts of H_2O_2 (the absence of Cl^- caused ~30% reduction in the H_2O_2 release by neutrophils; percentage of ^{51}Cr release: Cl^- absent = −0.5 ± 1.7, Cl^- present = 53.7 ± 3.2, n = 3, E:T = 2:1). Because the absence of Cl^- did not affect the MPO release by neutrophils (data not shown), the results suggest that Cl^- is required for the lytic reaction. Third, amino acids (L-alanine, L-serine, taurine), capable of rapidly reacting with HOCl without affecting neutrophil H_2O_2 release (data not shown and refs. 15, 27), inhibited CRBC lysis (Table 1). This indicates the requirement for HOCl. Taken together, the observations herein reported lead us to conclude that CRBC lysis by AGG.IgG-triggered neutrophils depends on the following reaction:

$$H_2O_2 + Cl^- \xrightarrow[H^+]{MPO} HOCl + H_2O.$$

Table 1. Effect of Scavengers of Oxygen Metabolites on the CRBC Lysis by Agg·IgG-Triggered Neutrophils

Additive	Percentage of ^{51}Cr Release*
None	54.3 ± 3.4
SOD, 300 U/mL	59.0 ± 3.2
Catalase, 4,000 U/mL	0.1 ± 0.6
Benzoate, 20 mmol/L	52.7 ± 4.4
Ethanol, 40 mmol/L	53.3 ± 2.2
Mannitol, 40 mmol/L	49.0 ± 1.0
NaN_3, 1 mmol/L	12.3 ± 2.8
Alanine, 20 mmol/L	8.3 ± 1.2
Serine, 20 mmol/L	8.0 ± 1.5
Taurine, 20 mmol/L	8.3 ± 2.0
$(NH_4^+)_2\ SO_4$, 10 mmol/L	2.3 ± 1.3

*Neutrophil:CRBC ratio = 1:1. Results are expressed as mean ± 1 SEM of three experiments.

The generated HOCl is capable of rapidly reacting with neutrophil-derived ammonia (NH_4^+) and taurine (Tau) to yield the respective nitrogen–chlorine (N–Cl) derivatives (NH_2Cl, TauNHCl, and possibly small amounts of $TauNCl_2$).[20,21,28-30] At present, HOCl and these N–Cl derivatives represent the main and well-characterized products of the neutrophil MPO-H_2O_2-Cl^- system: because of their oxidizing and chlorinating potential, they are candidates for the cytolysin role. Different observations, taken together, rule out the involvement of NH_2Cl, TauNHCl, and $TauNCl_2$ as lytic agents in our system. First, exogenous NH_4^+ [$(NH_4^+)_2SO_4$] inhibited CRBC lysis by neutrophils (Table 1), suggesting that lytic levels of NH_2Cl cannot be reached in our system even in the presence of an NH_4^+ surplus. Second, taurine, added in excess to favour TauNHCl formation,[20] inhibited CRBC lysis by neutrophils (Table 1): thus, the involvement of TauNHCl as cytolysin is unlikely. Third, CRBC treatment with DIDS to prevent the uptake of $TauNCl_2$ by CRBC themselves by the DIDS-inhibitable anion transport system,[11] inhibited the lysis by reagent $TauNCl_2$ (percentage of ^{51}Cr release: 22.4 ± 2.2 and 41.8 ± 2.1 for $CRBC^{DIDS}$ and native CRBC, respectively, $TauNCl_2$ = 50 nmol per well, n = 3), but not by neutrophils (percentage of ^{51}Cr release: 51.1 ± 1.7 and 53.3 ± 2.9 for $CRBC^{DIDS}$ and native CRBC, respectively, n = 3, E:T = 1:1): thus, $TauNCl_2$ should not be the cytolysin in our system. Fourth, no release of radioactivity was detected when ^{51}Cr-labeled neutrophils were incubated with unlabeled CRBC and Agg.IgG, as for cytolytic assays (percentage of ^{51}Cr release = 0.2 ± 0.3; parallel experiments with unlabeled neutrophils and labeled CRBC = 51.5 ± 3.2, n = 4, E:T = 1:1). Consequently, the generated cytolysin should be more effective against CRBC as compared with neutrophils. As shown in Fig 1, only HOCl displayed such a type of property. On the basis of these observations, we conclude that neutrophils exert lysis by means of HOCl or by means of

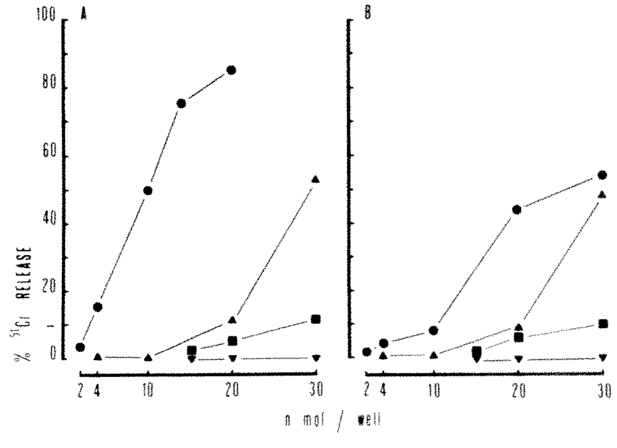

Fig 1. CRBC (A) and neutrophil (B) lysis by reagent HOCl (●), NH_2Cl (▲), TauNHCl (▼), and $TauNCl_2$ (■). CRBC = 10^5 cells per well, neutrophils = 10^5 cells per well. Results are expressed as mean of four experiments; standard deviations for each mean are all within 19% of the mean.

an unknown HOCl-derived compound with characteristics similar to HOCl.

When CRBC were replaced with HRBC, no lysis by neutrophils was observed (percentage of ^{51}Cr release: −0.1 ± 0.6, n = 3, E:T = 1:1). This finding cannot reflect a relative HRBC resistance to neutrophil-derived cytolysin because HRBC and CRBC were equally lysed by reagent HOCl (HOCl, dose required to cause 50% ^{51}Cr release, LD_{50}: 9.6 ± 0.3 and 9.8 ± 0.4 nmol per well for HRBC and CRBC, respectively, n = 3). Consequently, taking into account that HRBC are better equipped than CRBC with catalase (HRBC and CRBC catalase: 4,219 ± 82 and 592 ± 35 nmol H_2O_2/min/10^6 cells, respectively, n = 3; no difference in glutathione cycle components), we hypothesized that HRBC escape lysis by consuming (by catalase) so much neutrophil-derived H_2O_2 that HOCl cannot be produced in amounts sufficient to promote lysis. CRBC, endowed with sevenfold lower catalase content, only partially would limit the neutrophil lytic efficiency by a qualitatively similar process. To verify this hypothesis, we inhibited RBC H_2O_2-catabolizing pathways, catalase and glutathione cycle, by pulsing the cells with AT (HRBC^{AT} and CRBC^{AT} catalase = 215 ± 33 and 30 ± 15 nmol H_2O_2/min 10^6 cells, respectively; n = 3) and BCNU (RBC^{BCNU} = 100% inhibition of RBC GSSG-R), respectively. Then, we studied the effects of these target cell treatments on the neutrophil lytic efficiency. As depicted in Fig 2, the lysis of RBC^{BCNU} equalled that of native RBC, ruling out the involvement of glutathione cycle. Further, glucose-6-phosphate dehydrogenase (G6PD)-deficient HRBC, a natural model of glutathione cycle inefficiency,[31] were resistant to lysis by neutrophils (percentage of ^{51}Cr release: 0.2 ± 0.5, E:T = 1:1, n = 2). Conversely, the lysis of HRBC^{AT} and CRBC^{AT} was higher than that obtained with the respective native RBC (Fig 3A). In addition, lysis of RBC^{AT} was inhibited by catalase, azide, and amino acids (Table 2) in a way similar to that observed with native CRBC (Table 1): thus, neutrophils appear to lyse susceptible RBC (CRBC^{AT}, HRBC^{AT}, CRBC) by means of the same cytolysin. Because AT treatment of RBC did not modify per se the cell susceptibility to lysis by HOCl (Fig 3B), target cells can partially (CRBC) or completely (HRBC) restrain the HOCl-dependent neutrophil lytic activity through an AT-inhibitable, ie, catalase-mediated, process. This is consistent with our hypothesis. Further evidence for the hypothesis was obtained by measuring the effects of RBC on the extracellular availability of both H_2O_2 and HOCl, generated by neutrophils under experimental conditions similar to those used for lytic assays. Indeed, the recovery of H_2O_2 and HOCl from stimulated neutrophils was almost completely prevented by HRBC and partially reduced by CRBC, through a process significantly inhibited by pulsing RBC with AT but not with BCNU (Table 3). However, ~25% of the H_2O_2

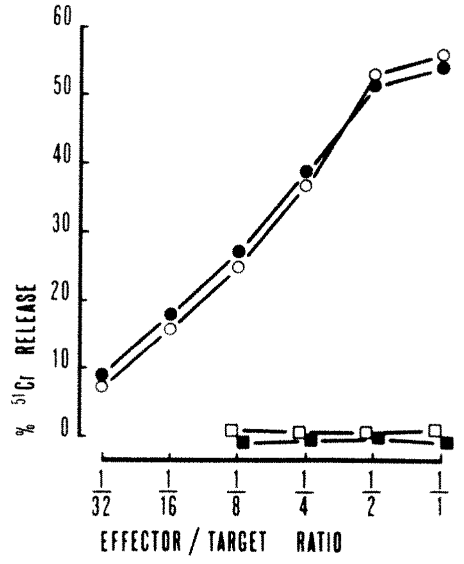

Fig 2. Lysis of CRBC (●), CRBC^{BCNU} (○), HRBC (■), and HRBC^{BCNU} (□) by Agg.IgG-triggered neutrophils. Treatment of RBC with BCNU-vehicle (ethanol 0.1%) alone did not affect their susceptibility to lysis. Each point represents the mean of three experiments; standard deviations for each mean are all within 15% of the mean.

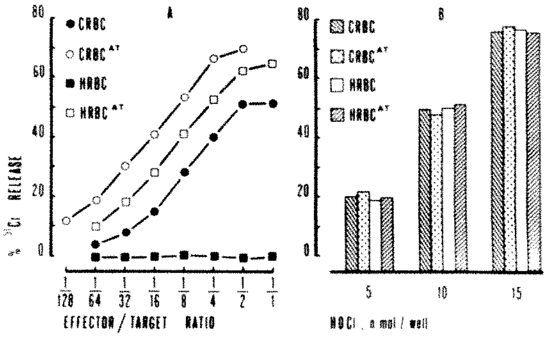

Fig 3. Lysis of CRBC, CRBC^{AT}, HRBC, and HRBC^{AT} by Agg.IgG-triggered neutrophils (A) and by reagent HOCl (B). Treatment of RBC with AT was performed in the presence of L-ascorbic acid as detailed in the Materials and Methods; L-ascorbic acid alone did not affect the RBC susceptibility to lysis by neutrophils and by HOCl. Experiments with HOCl were performed without centrifugation of the samples at the beginning of the assay. Each point represents the mean of three experiments; standard deviations for each mean are all within 18% of the mean. It is apparent that, for neutrophil-mediated lysis of CRBC, HRBC^{AT}, and CRBC^{AT} (A), the curves (ascending portion) are linear, $P < .001$, with similar slopes.

Table 2. Effect of Scavengers of Oxygen Metabolites on the Lysis of HRBC^{AT} and CRBC^{AT} by Agg·IgG-Triggered Neutrophils

Additive	Percentage of ^{51}Cr Release*	
	HRBC^{AT}	CRBC^{AT}
None	41.3 ± 2.0	42.0 ± 2.3
SOD, 300 U/mL	44.0 ± 2.5	45.3 ± 2.7
Catalase, 4,000 U/mL	0.4 ± 0.8	−0.1 ± 0.2
Benzoate, 20 mmol/L	41.3 ± 2.6	44.0 ± 2.3
Ethanol, 40 mmol/L	44.3 ± 2.8	44.0 ± 3.5
Mannitol, 40 mmol/L	42.3 ± 1.6	45.3 ± 3.7
NaN_3, 1 mmol/L	10.6 ± 2.2	10.0 ± 2.6
Alanine, 20 mmol/L	7.3 ± 1.6	6.3 ± 1.5
Serine, 20 mmol/L	7.7 ± 1.7	6.3 ± 1.5
Taurine, 20 mmol/L	5.7 ± 1.8	5.7 ± 1.7
$(NH_4^+)_2SO_4$, 10 mmol/L	3.7 ± 0.9	2.3 ± 0.9

*Neutrophil:HRBC^{AT} ratio = 1:8, neutrophil:CRBC^{AT} = 1:16. Results are expressed as mean ± SEM of three experiments.

Table 3. Recovery of Neutrophil-Derived H_2O_2 and HOCl in the presence and in the absence of RBC

	Extracellular Recovery†	
Neutrophils Incubated With*:	H_2O_2	HOCl
Medium alone	0.2 ± 0.1	0
Agg·IgG	17.6 ± 0.7	11.4 ± 0.8
Agg·IgG + HRBC	0.3 ± 0.1	0.4 ± 0.2
Agg·IgG + $HRBC^{AT}$	11.2 ± 0.9	6.9 ± 1.7
Agg·IgG + $HRBC^{BCNU}$	0.5 ± 0.3	0.4 ± 0.3
Agg·IgG + CRBC	7.5 ± 1.7	4.3 ± 0.4
Agg·IgG + $CRBC^{AT}$	13.6 ± 0.6	8.1 ± 1.7
Agg·IgG + $CRBC^{BCNU}$	7.7 ± 0.6	ND

ND, not done.

*Agg·IgG = 200 μg/mL; neutrophil:RBC ratio = 1:4.

†nmol/10^6 neutrophils per 30 minutes, mean ±1 SEM of three experiments.

could not be recovered in the presence of $CRBC^{AT}$, ie, cells virtually devoid of catalase activity (Table 3). This suggests that RBC can dissipate small amounts of H_2O_2 by means of nonenzymatic mechanisms, presumably hemoglobin.[21] Treatment of RBC with both AT and BCNU did not improve H_2O_2 recovery (data not shown).

When neutrophil lytic activity, calculated from Fig 3A, was plotted as a function of two closely correlated parameters (Fig 4A), ie, H_2O_2 and HOCl recovered from neutrophils in the presence of RBC (Table 3), positive linear relationships were found (Fig 4B). This finding, coupled with the evidence that RBC targets herein used have identical susceptibility to HOCl (Fig 3B) and with the results obtained by probing neutrophil cytolysis with chemical agents (Tables 1 and 2), strongly does argue for the concept that the extent of neutrophil-mediated lysis against different RBC (Fig 3A) strictly depends on the amount of HOCl produced and, in turn, on the amount of H_2O_2 available to generate HOCl itself. Under the experimental conditions used, target cell catalase activity behaves as a primary determinant of H_2O_2 availability to produce HOCl, ie, lysis. Indeed, each of these three parameters, as experimentally determined (Fig 3A, Table 3), correlates with the catalase content of RBC used (Fig 5). On the basis of these relationships, it can be calculated that a RBC catalase activity of ~1,200 nmol H_2O_2/min/10^6 cells represents the threshold below which cells can by lysed by Agg.IgG-triggered neutrophils, under the conditions herein used.

DISCUSSION

The interaction of neutrophil Fc receptor with Agg.IgG triggers two cell responses crucial to the lysis of surrounding target cells: production of H_2O_2 and secretion of MPO. These events are followed by the MPO-catalyzed oxidation of Cl^- ions by H_2O_2 to yield HOCl. The reaction presumably takes place in the extracellular spaces, although part of the secreted MPO remains absorbed to neutrophil surface, rather than being released as a free-enzyme into the medium.[29] Evidence for the requirement for each of these steps in the neutrophil-mediated cytolysis was provided by deleting each single component of the pathway: in fact, RBC lysis was inhibited by azide, catalase, Cl^--free medium, and amino acids, indicating the involvement of MPO, H_2O_2, Cl^-, and HOCl, respectively. This is in agreement with the capacity of neutrophil-free MPO-H_2O_2-Cl^- systems to lyse RBC.[32,33]

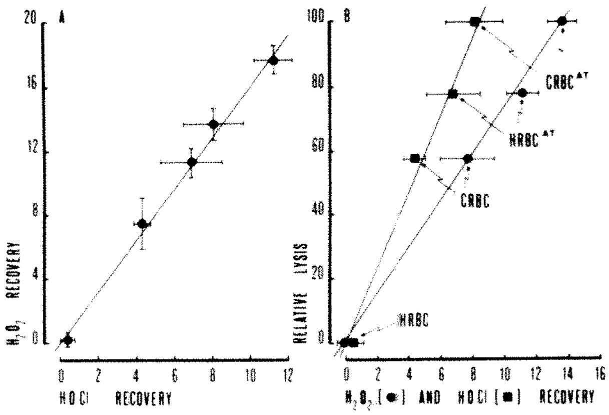

Fig 4. (A) Positive linear relationship ($P < .001$) between the amounts (nmol/10^6 cells per 30 minutes) of H_2O_2 and HOCl recovered from Agg.IgG-triggered neutrophils in the absence and in the presence of RBC, treated and not with AT (data from Table 3). (B) Positive linear relationships ($P < .01$) between the lytic efficiency displayed by Agg.IgG-triggered neutrophils against RBC, treated and not with AT (ordinate) and the H_2O_2 or HOCl recovered from Agg.IgG-triggered neutrophils in the presence of RBC (abscissa). The neutrophil lytic efficiency against different RBC is expressed as relative lysis, ie, as a percentage of the lysis observed with $CRBC^{AT}$ targets and taken as a maximum (100%). The data are calculated from the results depicted in Fig 3A (linear portion of the curves, effector:target ratio = 1:4). The values of H_2O_2 and HOCl recovery (nmol/10^6 cells per 30 minutes) are from Table 3.

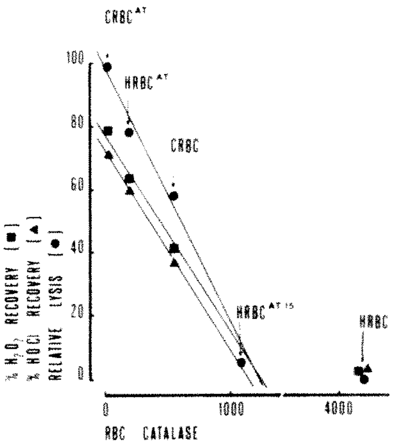

Fig 5. Negative linear relationships ($P < .05$) between the neutrophil lytic efficiency against different RBC, the HOCl recovery from neutrophils in the presence of different RBC, the H_2O_2 recovery from neutrophils in the presence of different RBC (ordinate), and the catalatic activity of the RBC used (abscissa). Neutrophil lytic efficiency is expressed as relative lysis, assuming the lysis of $CRBC^{AT}$ as 100% (see Fig 4; data are from Fig 3A; the point shown as $HRBC^{AT15}$ represents results obtained from additional experiments, performed with HRBC targets treated with AT for 15 minutes instead of 60 minutes). HOCl and H_2O_2 recovery are expressed as a percentage of the amounts of HOCl and H_2O_2 released by neutrophils incubated alone (data are from Table 3). Catalase activity of RBC is expressed as nmol H_2O_2/min/10^6 cells.

The subsequent events deserve special attention, as neutrophils were incubated in close contact with RBC. Under these conditions, the generated HOCl might collide with and lyse RBC, or it might act by reacting with a variety of neutrophil-derived *N*- compounds to yield N–Cl derivatives having chlorinating and oxidizing potential. In fact, activated neutrophils produce a heterogeneous pool of such N–Cl derivatives, mainly consisting of NH_2Cl and TauNHCl.[21,28-30] The chemical structure and the relative amounts of the remaining components of this N–Cl pool are only presumptive as yet. Therefore, HOCl, NH_2Cl, and TauNHCl, as well as $TauNCl_2$ recently found capable of lysing RBC,[11] have been studied as possible cytolysins in our system. The results suggest the following comments. First, the addition of NH_4^+ to enhance NH_2Cl production inhibited RBC lysis by neutrophils, suggesting that lytic concentrations of NH_2Cl cannot be achieved even in the presence of an NH_4^+ surplus. This is consistent with the data of Grisham et al.[21] In fact, by incubating stimulated neutrophils with RBC in suspension, these authors found that neutrophil-derived NH_2Cl oxidizes hemoglobin without producing lysis, through a reaction amplified by adding NH_4^+ to increase NH_2Cl production[21] and despite the capacity of NH_2Cl to limit neutrophil respiratory burst.[28] Second, the addition of taurine to activated neutrophils favors TauNHCl formation, preventing $TauNCl_2$ generation;[20] because added taurine inhibited RBC lysis by neutrophils, the involvement of TauNHCl as cytolysin can be ruled out. As regard $TauNCl_2$, the treatment of RBC with DIDS to inhibit the anion-transport system[10,11] reduced the lysis by $TauNCl_2$, confirming that lysis by $TauNCl_2$ is in part due to the uptake of this compound by RBC.[11] Nevertheless, DIDS-treated and native RBC were equally lysed by neutrophils. Thus, it seems unlikely that neutrophils use $TauNCl_2$ as cytolysin. Third, as neutrophils lysed RBC without "dying," the generated cytolysin is more effective against RBC as compared with neutrophils. Among the compounds tested (HOCl, NH_2Cl, TauNHCl, $TauNCl_2$), only HOCl displayed such property, and therefore it has characteristics compatible with the cytolysin role. Nevertheless, the neutrophil-free and the neutrophil-mediated cytolytic assay cannot be rationally compared in quantitative terms: in fact, in the former system, the cytolysin is added as a bolus and distributed throughout the assay volume, whereas in the latter one, it is generated as a flux and largely confined to the microenvironment of the effector–target pellet. Thus, on the basis of the observations listed earlier, we conclude that (a) The major and well-characterized N–Cl derivatives are not involved in the RBC lysis by neutrophils, under the present experimental conditions. However, these compounds can be responsible for toxic, ie, nonlytic, effects mediated by neutrophils toward RBC, as documented by other authors in a different experimental setting.[11,21,29] (b) The cytolysin, responsible for the RBC lysis by neutrophils, appears to be either HOCl or an unknown compound, derived from and with characteristics similar to HOCl. Further studies on this topic are needed, however, as, for example, Thomas et al have demonstrated that neutrophils chlorinate various "exogenous" amines to form potentially toxic N–Cl derivatives.[28] At present, the capacity of neutrophils to generate similar compounds from "endogenous" amines is unknown.

As stated above, NH_4^+- and taurine-N–Cl derivatives are not involved in the lysis. Thus, in agreement with other authors,[29] neutrophil-derived NH_4^+ and taurine act as down-regulators of the neutrophil lytic activity by trapping a fraction of the generated HOCl. Although RBC targets may contribute to such a type of down-regulation by releasing NH_4^+ in small quantities (ref. 21 and unpublished observations), they regulate neutrophil cytolytic activity primarily by interfering with HOCl production through the consumption of H_2O_2 by catalase. This conclusion is suggested by different findings. First, CRBC and HRBC limited the recovery of H_2O_2 and HOCl from neutrophils, activated under experimental conditions similar to those used for cytolytic assays. This finding is consistent with recent results obtained by Test and Weiss, using a different experimental system.[34] Second, both the HOCl-dependent neutrophil-mediated lysis and the recovery H_2O_2 and HOCl from neutrophils increased when native CRBC and HRBC were replaced with the respective AT-treated, ie catalase-depleted, cells. The neutrophil lytic activity and the H_2O_2–HOCl recovery strictly correlate with RBC catalase activity. It can be calculated that, under our experimental conditions, a RBC catalase activity of ~1,200 nmol $H_2O_2/min/10^6$ cells represents the threshold above which cells consume so much H_2O_2 that HOCl cannot be produced in amounts sufficient to promote lysis. Thus, CRBC and HRBC, respectively endowed with a catalase activity below and above this threshold, limit and respectively escape the HOCl-dependent neutrophil-delivered attack. In this connection, it seems suitable to remember the findings of Mandell[35] and those of Thomas and Aune.[36] The former found that *Staphylococcus aureus* catalase activity limits neutrophil-mediated killing and iodination, which is an MPO-dependent event.[37] The latter authors showed that killing of *Escherichia coli* by the MPO system depends on the competition for H_2O_2 between the bacteria and the MPO.

Although catalase and glutathione cycle probably play complementary roles in protecting RBC from hemolysis due to H_2O_2,[38] a protective role of RBC glutathione cycle in the present model of neutrophil-mediated lysis could not be demonstrated. In fact, no increase in the lysis by neutrophils was detected when native CRBC and HRBC were replaced with the respective cells treated with BCNU to inhibit GSSG-R activity. In addition, G6PD-deficient HRBC, a natural model of glutathione cycle inefficiency,[31] were as much resistant to neutrophil-mediated lysis as normal HRBC. Nevertheless, Weiss found that HRBC glutathione cycle, in combination with catalase, plays a protective role in a different model system.[39] Further, other target cells such as tumor cells and endothelial cells, can use their glutathione cycle to limit phagocyte-mediated lysis.[7,40,41] At present, the whole and detailed processing whereby catalase or glutathione cycle or both become operative under different experimental conditions is unclear.

In summary, our results suggest that RBC lysis by Agg.IgG-triggered neutrophils is due either to HOCl or to an unknown compound, derived from and with characteristics similar to HOCl. The lytic process is down-regulated by target cells and by neutrophils themselves. In fact, target cells appear to consume, primarily by catalase, neutrophil-derived H_2O_2 with a consequent impairment in the HOCl production. On the other hand, neutrophils release *N*-compounds, such as NH_4^+ and taurine, which dissipate a fraction of HOCl to yield N–Cl derivatives incapable of mediating lysis. It can be expected, however, that additional events play a role in limiting neutrophil lytic efficiency, for example, the inhibition of neutrophil respiratory burst by lipophilic N–Cl derivatives,[28] the consumption of H_2O_2 by neutrophils themselves,[34,42] the inactivation of MPO by HOCl,[43] and probably the target cell capacity of quenching MPO-derived cytolysins.[44,45] However, it seems that the inhibition of neutrophil cytolysin production by target cells remains a major determinant of the extent of the lysis. Although speculative, this mechanism might be envisaged as a natural means by which tissue cells can limit neutrophil-delivered attack, at least during inflammatory processes.

REFERENCES

1. Cochrane CG: Immunologic tissue injury mediated by neutrophilic leukocytes. Adv Immunol 9:97, 1968
2. Henson PM: Interaction of cells with immune-complexes: Adherence, release of constitutents, and tissue injury. J Exp Med 134:114, 1971
3. Fantone JC, Ward PA: Role of oxygen-derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am J Pathol 107:397, 1982
4. Dallegri F, Patrone F, Frumento G, Ballestrero A, Sacchetti C: Neutrophil-mediated cellular cytotoxicity triggered by immobilized aggregated IgG: An in vitro model of cell injury during immune complex disease. J Clin Immunol 4:439, 1984
5. Weissmann G, Smolen FE, Korchak HM: Release of inflammatory mediators from stimulated neutrophils. N Engl J Med 303:27, 1980
6. Babior BM: Oxidants from phagocytes: Agents of defense and destruction. Blood 64:959, 1984
7. Nathan CF, Arrick BA, Murray HW, De Sanctis NM, Cohn ZA: Tumor cell anti-oxidant defenses. J Exp Med 153:766, 1980
8. Dankberg F, Persidsky MA: A test of granulocyte membrane integrity and phagocytic function. Cryobiology 13:430, 1976
9. Margoliash E, Novogrodsky A: A study of the inhibition of catalase by 3-amino-1:2:3-triazole. Biochem J 68:468, 1958
10. Lynch RE, Fridovich I: Permeation of the erythrocyte stroma by superoxide radical. J Biol Chem 253:4697, 1978
11. Thomas EL, Grisham MB, Melton DF, Jefferson NM: Evidence for a role of taurine in the in vitro oxidative toxicity of neutrophils towards erythrocytes. J Biol Chem 260:3321, 1985
12. Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52:741, 1973
13. Dallegri F, Patrone F, Ballestrero A, Frumento G, Sacchetti C: Chemotactic peptide enhancement of PMA-triggered monocyte cytotoxicity. Clin Exp Immunol 57:717, 1984
14. Ruch W, Cooper PH, Baggiolini M: Assay of H_2O_2 production by macrophages and neutrophils with homovanillic acid and horse-radish peroxidase. J Immunol Methods 63:347, 1983
15. Weiss SJ, Klein R, Slivka A, Wei M: Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. J Clin Invest 70:598, 1982
16. Aune TM, Thomas EL: Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. Eur J Biochem 80:209, 1977
17. Bradley PP, Christensen RD, Rothstein G: Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood 60:618, 1982
18. Beutler E: Red cell metabolism. A manual of biochemical methods. Orlando, Fla, Grune & Stratton, 1975
19. Morris JC: The acid ionization constant of HOCl from 5 to 35 °. J Phys Chem 70:3798, 1966
20. Thomas EL, Jefferson MM, Grisham MB: Myeloperoxidase-catalyzed incorporation of amines into proteins: Role of hypochlorous acid and dichloramines. Biochemistry 21:2699, 1982
21. Grisham MB, Jefferson MM, Thomas EL: Role of monochloramine in the oxidation of erythrocyte hemoglobin by stimulated neutrophils. J Biol Chem 259:6766, 1984
22. Clark RA, Szot S: The myeloperoxidase-hydrogen peroxide-halide system as effector of neutrophil-mediated tumor cell cytotoxicity. J Immunol 126:1295, 1981
23. Halliwell B, Gutteridge JMC: Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219:1, 1984
24. Weiss SJ, Rustagi PK, Lo Buglio AF: Human granulocyte generation of hydroxyl radical. J Exp Med 147:316, 1978
25. Stelmaszynska T, Zgliczynski JM: Myeloperoxidase of human neutrophilic granulocytes as chlorinating enzyme. Eur J Biochem 45:305, 1974
26. Harrison JE, Schultz J: Studies on the chlorinating activity of myeloperoxidase. J Biol Chem 251:1371, 1976
27. Weiss SJ, Slivka A: Monocyte and granulocyte-mediated tumor cell destruction. A role for the hydrogen peroxide-myeloperoxidase-chloride system. J Clin Invest 69:255, 1982
28. Thomas EL, Grisham MB, Jefferson MM: Myeloperoxidase-dependent effect of amines on functions of isolated neutrophils. J Clin Invest 72:441, 1983
29. Grisham MB, Jefferson MM, Melton DF, Thomas EL: Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. J Biol Chem 259:10404, 1984
30. Test ST, Lampert MB, Ossanna PJ, Thoene JG, Weiss SJ: Generation of nitrogen-chlorine oxidants by human phagocytes. J Clin Invest 74:1341, 1984
31. Beutler E: Glucose-6-phosphate dehydrogenase deficiency, in Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds): Hematology. New York, McGraw-Hill, 1983, p 561
32. Vercellotti GM, Baken LS, Stroncek D, Jacob HS: Use of lysosome-free PMN cytoplasts ("neutroplasts") uncovers the critical role of myeloperoxidase for target cell lysis. Blood 64:204A, 1984 (abstr)
33. Dallegri F, Ballestrero A, Frumento G, Patrone F: Erythrocyte lysis by PMA-triggered neutrophil polymorphonuclears. Evidence for an hypochlorous acid-dependent process. Immunology 55:639, 1985
34. Test ST, Weiss SJ: Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. J Biol Chem 259:399, 1984
35. Mandell GL: Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. In vitro and in vivo studies with emphasis on staphylococcal-leukocyte interaction. J Clin Invest 55:561, 1975
36. Thomas EL, Aune TM: Cofactor role of iodide in peroxidase antimicrobial action against *Escherichia coli*. Antimicrob Agents Chemother 13:1000, 1978

37. Klebanoff SJ: Iodination of bacteria: A bactericidal mechanism. J Exp Med 126:1063, 1967

38. Chance B, Sies H, Boveris A: Hydroperoxide metabolism in mammalian organs. Physiol Rev 59:527, 1979

39. Weiss SJ: The role of superoxide in the destruction of erythrocyte targets by human neutrophils. J Biol Chem 255:9912, 1980

40. Harlan JM, Levine JD, Callahan KS, Schwartz BR, Harker LA: Glutathione redox cycle protects cultured endothelial cells against lysis by extracellularly generated hydrogen peroxide. J Clin Invest 73:706, 1983

41. Dallegri F, Frumento G, Patrone F: Mechanisms of tumor cell destruction by PMA-activated human neutrophils. Immunology 48:273, 1983

42. Nauseef WM, Metcalf JA, Root RK: Role of myeloperoxidase in the respiratory burst of human neutrophils. Blood 61:483, 1983

43. Naskalski JW: Myeloperoxidase inactivation in the course of catalysis of chlorination of taurine. Biochim Biophys Acta 485:291, 1977

44. Albrich JM, McCarthy A, Hurst JK: Biological reactivity of hypochlorous acid: Implications for microbicidal mechanisms of leukocyte myeloperoxidase. Proc Natl Acad Sci USA 78:210, 1981

45. Sepe SM, Clark RA: Oxidant membrane injury by the neutrophil myeloperoxidase system. II. Injury by stimulated neutrophils and protection by lipid-soluble antioxidants. J Immunol 134:1896, 1985

A Combination of a T Cell–Derived Lymphokine Differentiation-Inducing Activity and a Physiologic Concentration of Retinoic Acid Induces HL-60 to Differentiate to Cells With Functional Chemotactic Peptide Receptors

By Masue Imaizumi and Theodore R. Breitman

The human acute promyelocytic leukemia cell line HL-60 is induced by retinoic acid (RA) and *N,N*-dimethylformamide (DMF) to differentiate into cells having many of the functional and morphologic characteristics of mature granulocytes. With normal human phagocytic cells there is both superoxide anion (O_2^-) production and chemotaxis in response to chemoattractants such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). We have now found that although HL-60 cells induced with RA alone produce O^2_- in response to 12-0-tetradecanoyl-phorbol-13-acetate (TPA) they are deficient in FMLP-stimulated O_2^- production and chemotaxis. In contrast, HL-60 induced either with DMF or with a combination of 10 nmol/L RA and a T cell–derived lymphokine, differentiation-inducing activity (DIA), produce O_2^- and exhibit chemotaxis in response to FMLP. The basis for these results appears to be the concentration of cell surface chemotactic peptide receptors. Thus, untreated HL-60 and HL-60 induced with either RA alone or DIA alone do not have measurable levels of FMLP receptors, whereas HL-60 induced with a combination of RA and DIA has 5,400 receptors per cell. HL-60 induced with RA and DIA plus 1 μmol/L dexamethasone have 25,000 receptors per cell and have greater chemotactic activity than HL-60 induced with the combination of RA and DIA. Thus, differentiation of HL-60 to cells with many properties of normal phagocytes can be induced in vitro by physiologic substances.

IN THE PROCESS of differentiation, immature myelomonocytic cells undergo morphologic and cytochemical changes and acquire functions specialized for defense against microbial infection.[1] The human acute promyelocytic leukemia cell line HL-60 is a useful model to study the regulation and mechanism of differentiation in human myelomonocytic cells. HL-60 is induced to differentiate to granulocytelike cells by incubation with retinoic acid (RA), *N*, *N*-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO)[2,3] or into monocytelike/macrophagelike cells by incubation with 1-α,25-dihydroxyvitamin D_3 and 12-0-tetradecanoyl-phorbol-13-acetate (TPA).[4,5] Olsson et al[6] demonstrated that HL-60 are also induced to differentiate to monocytic cells by incubation with a physiologic concentration of RA[7] in combination with a human T cell–derived lymphokine (DIA, differentiation-inducing activity). Functional acquisitions of HL-60 induced with RA plus DIA include the expression of F_c receptors, immunophagocytosis, and superoxide anion (O_2^-) production activated by TPA.[8] In addition, these cells express the activities of two markers of monocytes/macrophages, nonspecific esterase and 5′-nucleotidase.[8]

In normal human phagocytic cells the production of O_2^- is one event in a cascade of reactions collectively called the respiratory burst. The respiratory burst can be elicited by opsonized particles[9] and chemoattractants such as synthetic *N*-formyl peptides.[10,11] Human phagocytic cells have surface receptors specific for *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), one of the most potent of the synthetic chemotactic peptides.[12,13] TPA, arachidonate, and other unsaturated fatty acids also stimulate the respiratory burst in human phagocytic cells but by pathways that are independent of the chemotactic peptide receptors.[14,15] Thus, the reaction pathways for O_2^- production in response to FMLP and TPA are not the same even though the same respiratory enzyme, NADPH oxidase, is activated.[16,17]

Chemotaxis, the directed migration of cells along a chemical gradient, is another important function of phagocytic cells and is required for the accumulation of effector cells at sites of inflammation. Phagocytic cells exhibit directed migration towards a variety of chemoattractants including *N*-formyl peptides,[10,11] and the presence of surface chemotactic peptide receptors is essential for this activity. Thus two functions of phagocytic cells, O_2^- production and chemotaxis, can be dependent on chemotactic peptide receptors.

Earlier studies showed increases in both the chemotactic peptide levels and chemotaxis of HL-60 induced with either DMF or DMSO[18,19] and that the glucocorticoid dexamethasone (DEX) increases chemotactic peptide receptor expression on differentiating HL-60 cells.[20,21] It was also shown that HL-60 cells induced by RA, with or without DEX, had no increase in the number of FMLP receptors and that RA blocked the induction of these receptors by DMF or DMSO.[20,22] These latter findings were of major interest because RA, of the many agents that induce differentiation of HL-60, has probably the most promise of being of use in the clinic. This is because it is active in vitro at physiologic concentrations,[2] induces differentiation of fresh human leukemia cells in primary culture,[23] and has been effective on patients with acute promyelocytic leukemia (APL).[24,25] The prospects that RA may produce mature phagocytes deficient in receptor-mediated O_2^- production and chemotaxis prompted the present investigation on the expression and functional activity of the chemotactic peptide receptors in HL-60 induced with a combination of a physiologic concentration of RA and the lymphokine DIA.

MATERIALS AND METHODS

Materials. RA, DEX, FMLP, nitroblue tetrazolium (NBT), bovine insulin, human transferrin, and cytochalasin B were obtained

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from Sigma Chemical Co, St Louis, Mo. Cytochalasin B was stored at −20 °C in ethanol at a concentration of 10 mg/mL. DMF was from Aldrich Chemical Co, Milwaukee, Wis. TPA was obtained from Pharmacia P-L Biochemicals, Inc, Milwaukee, Wis, and was stored at −20 °C in acetone at a concentration of 200 μg/mL. FMLP [phenylalanine-ring-2,6-^{3}H(N)] (48.3 Ci/mmol) was from NEN, Boston.

Cells. HL-60 was maintained in suspension culture in RPMI 1640 (Advanced Biotechnologies, Inc., Silver Spring, Md) supplemented with 10mmol/L 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) and 10% (vol/vol) fetal bovine serum (GIBCO, Grand Island, NY). The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air and split every week. Cell counts were determined by a Coulter Counter (Coulter Electronics, Hialeah, Fla), and viability was estimated by trypan blue dye exclusion. The HL-60 cell line was negative in tests for mycoplasma infection.

Induction of differentiation of HL-60. HL-60 was induced to differentiate with RA, DMF, and a T cell-derived lymphokine (DIA). HL-60 cells growing exponentially were harvested by centrifugation and resuspended at a density of 2.5 to 3.0 × 10^5/mL in a serum-free nutrient medium supplemented with 5 μg of insulin/mL and 5 μg of transferrin/mL.[26] After resuspension of HL-60 in this defined medium, induction of differentiation was initiated by adding various concentrations of RA, DMF, and DIA in the presence or absence of DEX. After incubation for four to five days as indicated, the capacity of the cells to reduce NBT in response to TPA or FMLP, expression of FMLP receptors, and chemotactic activity were studied. RA and DEX were dissolved in ethanol and diluted into the culture medium so that the final concentration of ethanol was less than 0.1%.

Assay for NBT reduction. The capacity of HL-60 to reduce NBT was assessed and expressed in two different ways: quantitatively as nanomoles of formazan produced per 10^6 cells and qualitatively as the percentage of formazan-positive cells. Viable cells (10^6) were harvested by centrifugation (400 g, seven minutes) and resuspended in 0.5 mL of RPMI 1640 containing 20% fetal bovine serum. This cell suspension was mixed with an equal volume of phosphate-buffered saline (PBS, 1.5 mmol/L KH_2PO_4, 8.1 mmol/L Na_2HPO_4, 136.9 mmol/L NaCl, pH 7.2) containing 1 mg of NBT/mL and either 200 ng of TPA/mL or various concentrations of FMLP. The reaction mixture was incubated at 37 °C and the reaction was terminated by cooling in an ice water bath. A 0.5-mL portion of the reaction mixture was applied to a microsample filtration device with glass fiber filters (Minifold, Schleicher & Schnell, Keene, NH) and washed with PBS and 0.01 *N* HCl. After drying in air, the filters were transferred to 4-mL glass vials, and 1 mL of DMF containing 10 μL of 1 *N* NaOH was added to each vial. The air in the vials was replaced with nitrogen to maintain the alkalinity. The vials were capped and then heated in a 50 °C water bath to increase the rate of elution of the formazan. The amount of formazan was determined from the absorbance at 710 nm using a molar absorption coefficient of 10^5/mol/L/cm. For determining the percentage of formazan-positive cells, cytospin slides were prepared from a portion of the chilled NBT reaction mixture and stained with Wright-Giemsa. A minimum of 200 cells were counted under light microscopy.

Assay of FMLP binding to cells. Viable cells (1.5 × 10^6) were incubated with various concentrations of ^{3}H-FMLP (48.3 Ci/mmol) in 200 μL of reaction buffer (PBS containing 0.9 mmol/L $CaCl_2$, 0.5 mmol/L $MgCl_2$, 0.5 mg bovine serum albumin/mL, 1 mmol/L NaN_3, and 50 μmol/L L-phenylalanine) at 25 °C for 45 minutes. At the end of the incubation period the reaction mixtures were cooled in an ice water bath, and 0.3 mL of cold reaction buffer was added to each reaction mixture. The reaction mixtures were filtered through glass fiber filters, and the filters were washed thoroughly with cold reaction buffer. The filters were dried and then counted in a β-liquid scintillation counter. All results are expressed as specific binding, defined as the difference between ^{3}H-FMLP binding in the absence and in the presence of 10 μmol/L unlabeled FMLP. Scatchard plot analysis of ^{3}H-FMLP binding was performed with the LIGAND computer program.[27]

Chemotaxis. Chemotaxis was assayed as described[13] with a 48-well microchemotaxis chamber (Neuro Probe, Inc, Cabin John, Md) and with polyvinylpyrrolindone-free polycarbonate filters (10 μm thick, 5-μm pores) (Nucleopore Corp, Pleasanton, Calif). The lower compartments of the wells were filled with 25 μL of chemotaxis buffer (Hanks' balanced salt solution supplemented with 10 mmol/L HEPES) containing various concentrations of FMLP. The upper compartments of the wells were filled with 50 μL of a cell suspension (2 × 10^6 viable cells/mL of chemotaxis buffer). The upper and lower compartments were separated by the filter. The chamber was incubated for 45 minutes at 37 °C in a humidified atmosphere of 5% CO_2 in air. After incubation, the cells on the upper surface of the filter (nonmigrated side) were removed by gently scraping the filter with a wiper blade. The filters were fixed in methanol and stained with Wright-Giemsa. The number of cells that migrated to the underside of the filter was evaluated with an Optomax System 4 Image Analyzer (Optomax, Inc, Hollis, NH).

DIA. Olsson et al.[28,29] reported that mitogen-stimulated peripheral blood human mononuclear cells released polypeptides (differentiation inducing factor(s)) that induce the maturation of HL-60. Breitman et al[8] proposed the more general term, differentiation inducing activity or DIA, because more than one polypeptide has this activity.[30,31] DIA was prepared from the conditioned medium of the T lymphocyte cell line HUT-102 and purified through the diethyl aminoethyl (DEAE) ion-exchange chromatography step essentially as described.[6] This preparation had a specific activity of approximately 14,000 U/mg protein and is devoid of other lymphokines such as colony-stimulating activity, interleukin 2, and γ-interferon.[6,30] One unit of DIA is defined as an amount per milliliter of serum-free defined medium containing 10 nmol/L RA that causes an increase of one in the percentage of NBT-positive HL-60 cells during a four-day incubation.

RESULTS

Growth and adhesiveness of HL-60 during induction with RA plus DIA. HL-60 cells growing in the presence of both RA and DIA change morphologically from round cells to cells with irregular shapes. This change is time dependent and is observed as early as day 1. By day 2 adhesive cells are observed. At day 4, in cultures grown in the presence of RA and DIA, 35% of the cells are adherent (Table 1). Untreated

Table 1. The combination of RA and DIA Promotes Adhesion of HL-60 Cells

Condition	Total Cells (× 10^5/mL)	Attached Cells (× 10^5/mL)	Percentage of Attached Cells
Untreated	10.9	0.18	1.6
10 nmol/L RA	10.3	0.19	1.9
DIA, 80 U/mL	12.7	0.24	1.9
10 nmol/L RA + DIA, 80 U/mL	8.4	2.94	35.0

Cells (3 × 10^5/mL), in a total volume of 2.5 mL of defined medium, were grown in wells of a 6-well dish for four days. The medium containing the suspended cells was removed, and the surface of the dish was washed gently with PBS. Attached cells were removed with a cell scraper.

cells and cells grown in the presence of RA alone or DIA alone are not adherent.

FMLP- and TPA-stimulated NBT reduction of HL-60 induced by either RA, DMF, or RA plus DIA. The report of Skubitz et al[20] showed that HL-60 induced with DMF had an increased concentration of FMLP receptors whereas HL-60 induced with RA had no increase and in fact may have had a decrease. In normal human granulocytes both FMLP and TPA stimulate oxidative metabolism with the production of O_2^- as measured by the reduction of ferricytochrome c.[16] We reasoned that FMLP-stimulated O_2^- production, as measured by the reduction of NBT, would correlate with the concentration of FMLP receptors on HL-60 induced under different conditions. DMF, in a dose-dependent manner, induces HL-60 to differentiate to cells that reduce NBT in response to either TPA or FMLP (Fig 1). There is a greater stimulation with TPA than with either 100 nmol/L or 1 μmol/L FMLP. A greater stimulation of oxidative metabolism by TPA than by FMLP has also been observed for normal human granulocytes.[16] In contrast to DMF, only a small percentage of RA-induced cells reduce NBT in response to FMLP even though the population is quite active in response to TPA (Fig 2). A peak response to FMLP occurs with cells induced with approximately 100 nmol/L RA; concentrations of RA greater than 100 nmol/L are inhibitory.

The response of DMF-induced and RA-induced HL-60 to FMLP stimulation of NBT reduction appeared at this stage of our study to correlate qualitatively with the reported relative levels of chemotactic peptide receptors. The finding that HL-60 induced with a combination of RA and DIA actively reduced NBT when stimulated with FMLP indicated that these cells had increased concentrations of FMLP receptors (Fig 3). In the presence of a physiologic concentration of RA (10 nmol/L) there is a DIA concentration-dependent increase in the percentage of cells reducing NBT in response to either TPA or FMLP, with the response to TPA being greater than to FMLP. Cells incubated with DIA alone or RA alone were essentially inactive to stimulation by FMLP. Cells incubated with DIA alone responded to TPA with values for differentiation of approximately 8% (Fig 3).

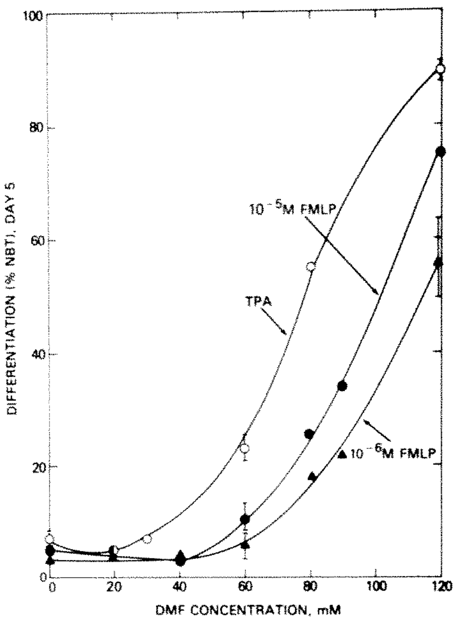

Fig 1. TPA- and FMLP-stimulated reduction of NBT by DMF-induced cells. HL-60 cells were grown for five days in defined medium containing various concentrations of DMF. Cells were then incubated at 37 °C for 25 minutes with 0.5 mg NBT/mL and either 200 ng TPA/mL (○), 1 μmol/L FMLP (▲), or 10 μmol/L FMLP (●). The extent of NBT reduction is expressed as the percentage of formazan-positive cells on cytospin slides. Each value with bars is the mean ± SEM of at least three experiments. Values without bars are from one experiment.

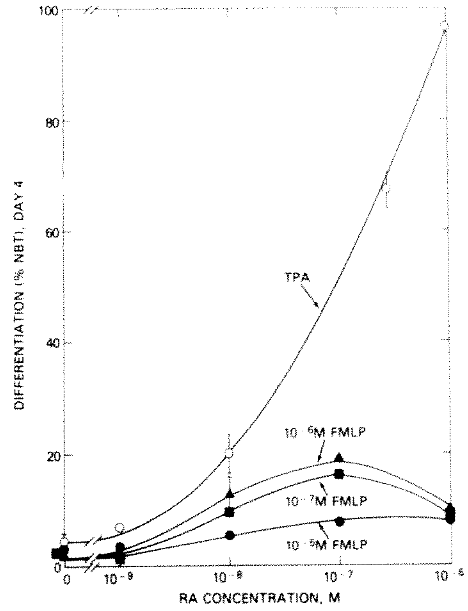

Fig 2. TPA- and FMLP-stimulated reduction of NBT by RA-induced cells. HL-60 cells were grown for four days in defined medium containing various concentrations of RA. Cells were then incubated at 37 °C for 25 minutes with 0.5 mg NBT/mL and either 200 ng TPA/mL (○), 1 μmol/L FMLP (▲), 10 μmol/L FMLP (●), or 100 nmol/L FMLP (■). The extent of NBT reduction is expressed as the percentage of formazan-positive cells on cytospin slides. Values with and without bars are as described in Fig 1.

Quantitation of formazan production. In the aforementioned experiments, a qualitative NBT assay was used that determines the percentage of cells in a population that have a microscopically determined level of cell-associated formazan. This assay does not give information on how much formazan is produced except in subjective terms. To obtain this information we have recently adapted (unpublished observations, 1984) the quantitative NBT test of Baehner and Nathan[32] to measure the quantity of NBT that is reduced. This assay, as with the more commonly used reduction of ferricytochrome c, gives information on the activity of the total population of cells. The data in Fig 4 show that HL-60 grown for four days in the presence of a combination of 10 nmol/L RA and various concentrations of DIA have a greater capacity to reduce NBT in response to a stimulation by FMLP than either control cells, cells incubated with 10 nmol/L RA alone, or cells grown with DIA alone. In addition, exposure of these cells to cytochalasin B

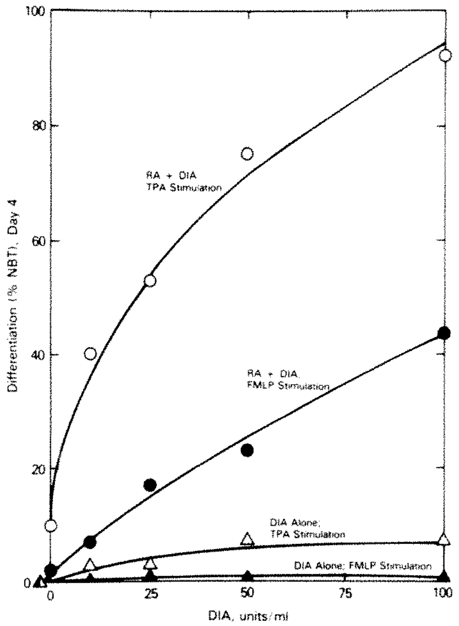

Fig 3. TPA- and FMLP-stimulated reduction of NBT by HL-60 induced with a combination of RA and DIA. Cells were grown for four days in defined medium containing 10 nmol/L RA and various concentrations of DIA. Cells were then incubated at 37 °C for 25 minutes with 0.5 mg NBT/mL and either 200 ng TPA/mL or 1 µmol/L FMLP. The extent of NBT reduction is expressed as the percentage of formazan-positive cells on cytospin slides.

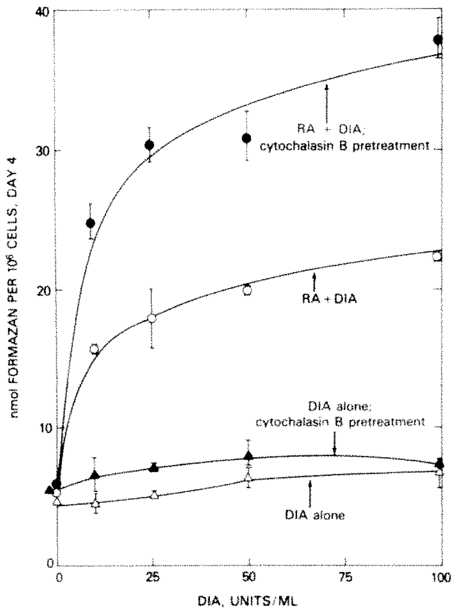

Fig 4. Enhancement by cytochalasin B of FMLP-stimulated production of formazan by HL-60 induced with a combination of RA and DIA. Cells were grown for four days in defined medium containing the indicated concentration of DIA with and without 10 nmol/L RA. A portion of each culture was incubated for 20 minutes with 5 µg cytochalasin B/mL (pretreatment) and then the cells (pretreated and nonpretreated) were incubated at 37 °C for 25 minutes with 0.5 mg NBT/mL and 1 µmol/L FMLP. Values are the means of two experiments. Bars are the range.

immediately prior to the NBT assay results in a marked increase in formazan production. A similar effect of cytochalasin B has been reported on FMLP-stimulated O_2^- production by normal granulocytes.[16]

Expression of FMLP receptors. The increased response to FMLP of HL-60 cultured in the presence of RA and DIA suggested that these cells had increased concentrations of FMLP receptors. Measurements of the specific binding of FMLP show that HL-60 grown for four days in the presence of RA and DIA have a much greater capacity to bind FMLP than untreated cells or cells grown with 300 nmol/L RA (Fig 5). In separate experiments incubation with DIA alone did not result in an increase of FMLP binding (data not shown). In agreement with the findings of Brandt et al[21] and Skubitz et al[20] for DMF- and DMSO-induced cells, the addition of 1 µmol/L DEX to the induction medium results in a marked increase of specifically bound FMLP (Fig 5). From a Scatchard plot analysis (Fig 6) there are 5,400 receptors per cell for cells induced with RA plus DIA and an increase of approximately fivefold to 25,000 receptors per cell for cells induced with added DEX. The calculated K_D values are 19.73 nmol/L and 11.37 nmol/L, respectively. Thus, treatment with DEX results in an increased number of chemotactic peptide receptors with an increased affinity for FMLP.

It has been reported that DEX has little effect on DMF-induced or DMSO-induced differentiation of HL-60 assessed by cell morphology[20,21] or by the ability of the cells to reduce NBT when stimulated by TPA.[20] However, FMLP-stimulated NBT reduction was not examined. NBT reduction in response to FMLP was measured in HL-60 cells induced for four days in the presence of 10 nmol/L RA and

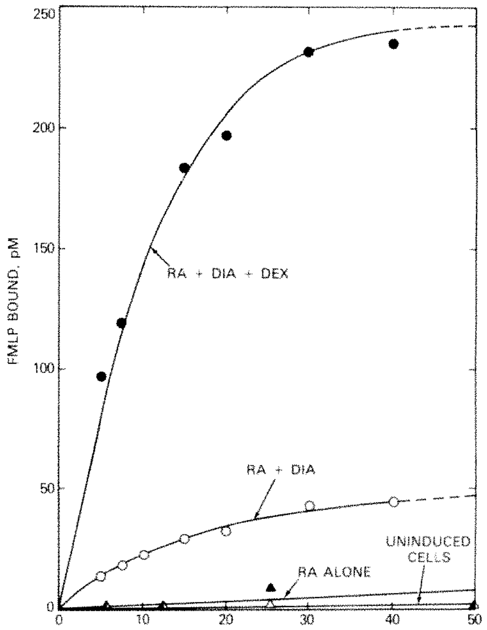

Fig 5. Increased specific binding of FMLP to HL-60 grown for four days in the presence of 10 nmol/L RA and DIA (80 U/mL) with (●) and without (○) 1µmol/L DEX compared with untreated cells (△) and cells grown with 300 nmol/L RA (▲). For each condition there were 7.5 × 10⁶ cells per mL.

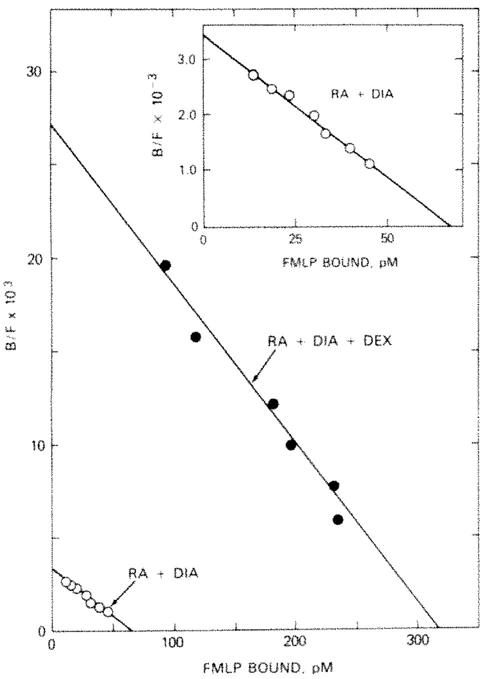

Fig 6. Scatchard plot analysis of FMLP binding to HL-60 cells. Data from Fig 5 for the conditions of growth in the presence of 10 nmol/L RA and DIA (80 U/mL) with (●) and without (○) DEX were analyzed with the LIGAND computer program.[27] The lines were drawn from the computed values for K_D and receptor concentration. The insert is an enlargement of the data for the RA plus DIA condition.

100 U DIA/mL with and without 1 μmol/L DEX. It was found that at FMLP concentrations greater than 100 nmol/L only approximately 1.2-fold more formazan was produced by cells induced in the presence of DEX (data not shown). In a two-tailed *t* test this difference had a *P* value of .05 and is not statistically significant. These results may indicate that a minimum receptor concentration of less than 5,400 per cell is required for maximum chemotactic peptide stimulation of O_2^- production.

Chemotaxis of HL-60. HL-60 induced by RA plus DIA exhibits chemotactic peptide concentration-dependent migration (Fig 7). Cells induced in the presence of DEX are more active. The percentage of total cells migrating in response to the optimal concentration of 100 nmol/L FMLP increased from 26% for cells induced in the absence of DEX to 47% for cells induced in the presence of 1 μmol/L DEX. This difference has a *P* value of .01 and is considered significant. However, most if not all of the increased response in DEX-treated cells is probably a result of the increased nondirected migration observed in the absence of FMLP (Fig 7). Untreated cells, cells induced with RA alone (Fig 7), and cells cultured with DIA alone (data not shown) were essentially unresponsive to FMLP.

DISCUSSION

RA induces HL-60 to differentiate to cells with granulocytelike morphology and with the ability to express reactions associated with the respiratory burst, such as O_2^- production, NBT reduction, and an increased hexose monophosphate shunt (HMPS) activity.[8] However, although these HL-60 cells actively produce O_2^- in response to TPA (measured by NBT reduction), they are relatively inactive in response to FMLP, a potent chemotactic peptide (Fig 2). In contrast, HL-60 induced to differentiate with DMF is active in reducing NBT in response to either TPA or FMLP (Fig 1). This difference in the response of HL-60 to FMLP was expected from previous studies showing that the number of chemotactic peptide receptors on HL-60 is increased by treatment with DMF or DMSO[18-21] and does not change or is decreased by RA treatment.[20]

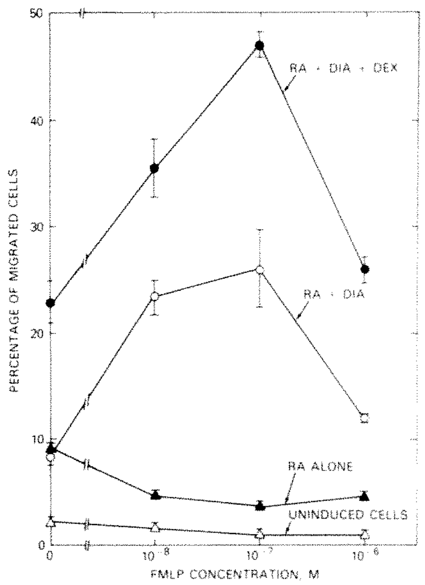

Fig 7. Chemotaxis of HL-60 cells in response to FMLP. Conditions for induction and symbols are the same as those described in Fig 5. Each point is the mean ± SEM of three independent determinations.

A lower response to FMLP than to TPA is observed for HL-60 induced with increasing concentrations of DMF (Fig 1). A similar difference in the response to these soluble mediators has been found for normal human granulocytes[16] for which evidence has been presented that it is a consequence of different transductional mechanisms operating under different controls and is not due to an activation of different respiratory enzymes.[17] The low response of RA-induced HL-60 to FMLP (Fig 2) is explained by the very low levels of FMLP binding to these cells (Fig 5). These results are in agreement with Skubitz et al[20] who, in addition, found that RA concentrations greater than 10 nmol/L blocked induction by DMF of FMLP receptors. Therefore, the increase followed by a decrease in the percentage of cells reducing NBT in response to 100 nmol/L and 1 μmol/L FMLP (Fig 2) may be the sum of inductive and inhibitory effects of various RA concentrations. At low concentrations of RA (10 nmol/L) there is primarily induction, whereas at higher concentrations (>100 nmol/L) the inhibitory effect dominates. This inhibitory effect of RA appears to be confined to FMLP receptor-mediated O_2^- production since TPA-stimulation was quite active (Fig 2). Thus, it appears that HL-60 induced with RA develops the transductional mechanism that can be stimulated by TPA but does not express the transductional mechanism that is stimulated by FMLP because of a deficiency of chemotactic peptide receptors. In a related study, Stendahl et al[22] have reported recently that RA-induced HL-60, compared with DMSO-induced HL-60, is deficient in receptors for FMLP and in both FMLP-stimulated calcium uptake and FMLP-stimulated O_2^- production measured by ferricytochrome c reduction. In addition, these workers found that 500 nmol/L RA inhibited DMSO-induced increases in these parameters. IgG-mediated phagocytosis and leukotriene B–stimulated calcium uptake were not affected by RA, and C3b-mediated phagocytosis was partially decreased by RA. Thus, pharmacologic concentrations of RA result in a maturation of HL-60 to cells that are deficient in some characteristics of mature normal phagocytes. The mechanism by which RA inhibits the development of FMLP receptors is not known.

Although induction of HL-60 with RA alone yields cells that are deficient in chemotactic peptide receptor concentration and function, our results indicate that the combination of a T cell–derived lymphokine (DIA) and a physiologic concentration of RA (10 nmol/L) induces HL-60 to differentiate to cells with an increased number of FMLP receptors (Figs 5 and 6). These receptors are functional as shown by FMLP-dependent O^2_- generation (Figs 3 and 4) and FMLP-directed migration (Fig 7). Pretreatment of these cells with cytochalasin B increased FMLP-stimulated NBT reduction by approximately 1.5-fold (Fig 4). This effect is seen also with normal human granulocytes.[16]

There have been two reports on the effect of DEX on the properties of FMLP receptors expressed on HL-60 induced by DMF or DMSO.[20,21] These studies indicate that DEX induces a three- to fourfold increase in receptor number without a significant change of affinity. Our results on RA plus DIA agree with these reports regarding receptor number but indicate that there is a twofold increase in the affinity of the chemotactic peptide receptor induced in the presence of DEX (Fig 6). In normal human monocytes there are two populations of monocytes in regard to chemotactic activity and expression of FMLP receptors; one subpopulation migrates to FMLP and has saturable binding sites for FMLP. The other subpopulation does not migrate and exhibits little chemotactic peptide binding.[13] In contrast to monocytes, migrating and nonmigrating populations of normal human granulocytes show identical binding characteristics for FMLP.[33] We have not studied whether there are subpopulations with heterogenous chemotactic activity and receptor expression in the monocytelike/macrophagelike HL-60 cells induced by RA plus DIA.

Chemotaxis is an integrated function of phagocytic cells, consisting of various properties such as adhesiveness, orientation, presence of chemoattractant receptors, and directed mobility. Several groups have shown that HL-60 induced by DMF and DMSO exhibits an increase of chemotactic activity.[18,19,34] In this study we have shown that HL-60 cells induced by RA in combination with DIA exhibit not only expression of FMLP receptors but also chemotactic activity (Fig 7). Furthermore, induction in the presence of DEX increases both the number of receptors and chemotactic activity (Figs 6 and 7). Alteri and Leonard[35] found that normal human peripheral monocytes exhibit a maximal chemotactic response at concentrations of 10 nmol/L to 100 nmol/L FMLP. In contrast, the highest chemotactic activity of normal granulocytes is at 1 μmol/L FMLP[33] a concentration that markedly inhibits chemotaxis of monocytes.[35] HL-60 induced by RA plus DIA, with and without DEX, exhibited maximal chemotaxis at concentrations of 10 nmol/L to 100 nmol/L FMLP, and 1 μmol/L FMLP was inhibitory (Fig 7).

Recently, there has been interest in the possibility that differentiation inducers may have use in the treatment of some malignancies. This concept is predicated on the belief that some malignancies are a result of a block in differentiation that if relieved would result in a more differentiated and therefore more benign malignancy. As a concept for therapy this approach holds the further promise that induction of differentiation could not only relieve the tumor burden but also increase the number of functional cells, the absence of which is, at least for some malignancies, a major complication. HL-60 has been a useful model system in the search for substances that are active as inducers of differentiation. Of the many compounds that induce differentiation of HL-60, RA has probably the most promise of being of use in the clinic. This is because it is active at physiologic concentration in vitro,[2] has been shown to induce differentiation of fresh human leukemia cells in primary culture,[23,25,36,37] and has been reported to be effective on patients with APL.[24,25] However, while RA as a sole agent and at pharmacologic concentrations of approximately 1 μmol/L may be a good therapeutic agent, experiments in vitro indicate that the combination of RA and either cyclic adenosine monophosphate–inducing agents or lymphokines act synergistically to induce differentiation of HL-60 as well as other myelomonocytic cell lines.[6,8,38] In addition, combinations of RA and DIA synergistically induce differentiation of fresh human leukemia cells in primary culture.[8] To the extent that it is possible, results in vitro should suggest treatments in vivo. To this end we have been interested in determining what induction conditions result in the most normal mature HL-60.

Previous studies have indicated that combinations of RA and the lymphokines DIA or γ-interferon differentiate HL-60 to monocytelike cells.[8,38] These cells exhibit immunophagocytosis, increases in F_c receptors, reduction of NBT, 5′-nucleotidase activity, nonspecific esterase activity, and antibody-dependent cellular cytotoxicity. One of the more important functions of the normal phagocytic cell is its ability to seek out and kill invading organisms. Our results show that HL-60 induced with a combination of 10 nmol/L RA and DIA mature to cells that show adherence (Table 1) and increases in FMLP receptors that are functional for both O_2^- production and directed migration. We feel that it is important that this differentiation occurs in response to two physiologic substances and that one of the components of this combination, RA, is at the physiologic plasma concentration of approximately 10 nmol/L.[7] The physiologic concentration of DIA is unknown, but it is probable that this substance(s) plays a role, along with RA and other factors, in normal hematopoiesis.

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REFERENCES

1. Zakhireh B, Root RK: Development of oxidase activity by human bone marrow granulocytes. Blood 54:429, 1979

2. Breitman TR, Selonick SE, Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77:2936, 1980

3. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC: Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci USA 75:2458, 1978

4. Rovera G, Santoli D, Damsky C: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci USA 76:2779, 1979

5. McCarthy DM, San Miguel JF, Freake HC, Green PM, Zola H, Catovsky D, Goldman JM: 1,25-Dihydroxyvitamin D_3 inhibits proliferation of human promyelocytic leukemia (HL-60) cells and induces monocyte-macrophage differentiation in HL-60 and normal human bone marrow cells. Leuk Res 7:51, 1983

6. Olsson IL, Breitman TR, Gallo RC: Priming of human myeloid leukemic cell lines HL-60 and U-937 with retinoic acid for differentiation effects of cyclic adenosine 3':5'-monophosphate–inducing agents and a T-lymphocyte–derived differentiation factor. Cancer Res 42:3928, 1982

7. DeRuyter MG, Lambert WE, Deleenheer AP: Retinoic acid: An endogenous compound of human blood. Unequivocal demonstration of endogenous retinoic acid in normal physiological conditions. Anal Biochem 98:402, 1979

8. Breitman TR, Keene BR, Hemmi H: Retinoic acid–induced differentiation of fresh leukaemia cells and the human myelomonocytic leukaemia cell lines, HL-60, U-937, and THP-1. Cancer Surveys 2:263, 1983

9. Babior BM: Oxygen-dependent microbial killing by phagocytes. N Engl J Med 298:659, 1978

10. Schiffmann E, Corcoran BA, Wahl SM: *N*-formylmethionyl peptides as chemoattractants for leukocytes. Proc Natl Acad Sci USA 72:1059, 1975

11. Showell HJ, Freer RJ, Zigmond SH, Schiffmann E, Aswanikumar S, Corcoran B, Becker EL: The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. J Exp Med 143:1154, 1976

12. Williams TL, Snyderman R, Pike MC, Lefkowitz RJ: Specific receptor sites for chemotactic peptides on human polymorphonuclear leukocytes. Proc Natl Acad Sci USA 74:1204, 1977

13. Falk W, Harvath L, Leonard EJ: Only the chemotactic subpopulation of human blood monocytes expresses receptors for the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine. Infect Immun 36:450, 1982

14. DeChatelet LR, Shirly PS, Johnston RB Jr: Effects of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. Blood 47:545, 1976

15. Badwey JA, Curnutte JT, Karnovsky ML: *cis*-Polyunsaturated fatty acids induce high levels of superoxide production by human neutrophils. J Biol Chem 256:12640, 1981

16. Lehmeyer JE, Snyderman R, Johnston RB Jr: Stimulation of neutrophil oxidative metabolism by chemotactic peptides: Influence of calcium ion concentration and cytochalasin B and comparison with stimulation with phorbol myristate acetate. Blood 54:35, 1979

17. McPhail LC, Snyderman R: Activation of the respiratory burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli. J Clin Invest 72:192, 1983

18. Fontana JA, Wright DG, Schiffman E, Corcoran BA, Deisseroth AB: Development of chemotactic responsiveness in myeloid precursor cells: Studies with a human leukemia cell line. Proc Natl Acad Sci USA 77:3664, 1980

19. Niedel J, Kahane I, Lachman L, Cuatrecasas P: A subpopulation of cultured human promyelocytic leukemia cells (HL-60) displays the formyl peptide chemotactic receptor. Proc Natl Acad Sci USA 77:1000, 1980

20. Skubitz KM, Zhen Y, August JT: Dexamethasone synergistically induces chemotactic peptide receptor expression in HL-60 cells. Blood 59:586, 1982

21. Brandt SJ, Barnes KC, Glass DB, Kinkade JM Jr: Glucocorticoid-stimulated increase in chemotactic peptide receptors on differentiating human myeloid leukemia (HL-60) cells. Cancer Res 41:4947, 1981

22. Stendahl O, Andersson T, Coble BI, Dahlgren C, Lew D: Selective modulation of a chemotactic receptor in HL-60 cells by retinoic acid, in Saurat J (ed): Retinoids: New Trends in Research and Therapy. Retinoid Symposium, Geneva, 1984. Basel, Switzerland, Karger, 1985, p 260

23. Breitman TR, Collins SJ, Keene BR: Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. Blood 57:1000, 1981

24. Nilsson B: Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukaemia. Br J Haematol 57:365, 1984

25. Flynn PJ, Miller WJ, Weisdorf DJ, Arthur DC, Brunning R, Branda RF: Retinoic acid treatment of acute promyelocytic leukemia: In vitro and in vivo observations. Blood 62:1211, 1983

26. Breitman TR, Keene BR, Hemmi H: Studies of growth and differentiation of human myelomonocytic leukemia cell lines in serum-free medium, in Barnes DW, Sirbasku DA, Sato GH (ed): Methods for Serum-Free Culture of Neuronal and Lymphoid Cells. New York, Liss, 1984, p 215

27. Munson PJ, Rodbard D: LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220, 1980

28. Olsson I, Olofsson T: Induction of differentiation in a human promyelocytic leukemic cell line (HL-60). Production of granule proteins. Exp Cell Res 131:225, 1981

29. Olsson I, Olofsson T, Mauritzon N: Characterization of mononuclear blood cell–derived differentiation inducing factors for the human promyelocytic leukemia cell line HL-60. J Natl Cancer Inst 67:1225, 1981

30. Takei N, Takeda K, Konno K: The role of interferon-γ in induction of differentiation of human myeloid leukemia cell lines, ML-1 and HL-60. Biochem Biophys Res Commun 124:100, 1984

31. Leung K, Chiao JW: Human leukemia cell maturation induced by a T-cell lymphokine isolated from medium conditioned by normal lymphocytes. Proc Natl Acad Sci USA 82:1209, 1985

32. Baehner RL, Nathan DG: Quantitative nitroblue tetrazolium test in chronic granulomatous disease. N Engl J Med 278:971, 1968

33. Harvath L, Leonard EJ: Two neutrophil populations in human blood with different chemotactic activities: Separation and chemoattractant binding. Infect Immun 36:443, 1982

34. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC: Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethyl sulfoxide. J Exp Med 149:969, 1979

35. Alteri E, Leonard EJ: *N*-formylmethionyl-leucyl-[^{3}H]phe-

nylalanine binding, superoxide release and chemotactic responses of human blood monocytes that repopulate the circulation during leukapheresis. Blood 62:918, 1983

36. Honma Y, Fujita Y, Kasukabe T, Hozumi M, Sampi K, Sakurai M, Tsushima S, Nomura H: Induction of differentiation of human acute nonlymphocytic leukemia cells in primary culture by inducers of differentiation of human myeloid leukemia cell line HL-60. Eur J Cancer Clin Oncol 19:251, 1983

37. Honma Y, Fujita Y, Kasukabe T, Hozumi M, Sampi K, Sakurai M, Tsushima S, Nomura H: Differentiation in vitro of human myelogenous leukemia cells from patients in relapse. Gann 75:518, 1984

38. Hemmi H, Breitman TR: Monocytic differentiation of human myelomonocytic leukemia cell lines induced by T-lymphocyte derived differentiation inducing activity. Tissue Culture (Japan) 10:185, 1984 (abstr)

Proteolytic Cleavage of Human von Willebrand Factor Induced by Enzyme(s) Released From Polymorphonuclear Cells

By E. Anne Thompson and Margaret A. Howard

In vivo fragmentation of the von Willebrand factor antigen (vWF:Ag) molecule has been demonstrated on radio-crossed immunoelectrophoresis (CIE) in the plasma from patients with disseminated intravascular coagulation, in factor VIII concentrates, and in normal serum. Experiments reported here show that polymorphonuclear (PMN) cells contain a non-calcium-dependent protease(s) that when released and incubated with vWF:Ag results in an additional vWF:Ag peak on radio-CIE.

Production of fragments of vWF:Ag by incubation with PMN cells occurred in a time-dependent manner. The protease(s) responsible was inhibited by diisopropyl fluorophosphate, soybean trypsin inhibitor, and aprotinin, but not by benzamidine, azide, epicron, or hirudin. Citrate, EDTA, and leupeptin also had no effect on the PMN cell enzyme's activity, indicating that the enzyme(s) is not calcium dependent. The PMN cell enzyme responsible for vWF:Ag fragmentation is located intracellularly and released by freeze-thaw lysis or cell activation by calcium or the calcium ionophore A23187.

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PROCOAGULANT factor VIII (VIII:C), measured as coagulant activity in a clotting assay, circulates as a complex with von Willebrand factor (vWF), an essential requirement for platelet adhesion to injured vessels. vWF is measured functionally in vitro as ristocetin cofactor activity (RCof) and immunologically as von Willebrand factor antigen (vWF:Ag).

A number of enzymes, namely plasmin,[1] trypsin,[2] chymotrypsin,[3] and an elastaselike protease[4] derived from granulocytes reduce RCof activity and produce an apparent increase in the vWF:Ag level.[1-4] The apparent increase in the vWF:Ag level has been suggested to be due to a decreased molecular size as a result of proteolytic cleavage of the vWF molecule. The smaller protein migrates more rapidly in the immunoelectrophoretic assay used for vWF:Ag quantitation, giving a falsely elevated level. Other enzymes such as thrombin and activated protein C that inactivate VIII:C[3,5,6] have no known effect on the structure or function of the RCof/vWF:Ag molecule.

Recent evidence indicates that the vWF molecule undergoes fragmentation both in vivo and in vitro. An antigen showing partial immunologic identity to vWF:Ag and present in the plasma from patients with disseminated intravascular coagulation (DIC) has been described.[7-9] This antigen is also present in all normal sera prepared by allowing whole blood to clot, but is not present in recalcified plasma or platelet-rich plasma.[9] However, it has been reported that cleavage of vWF:Ag by a platelet calcium-activated protease (CAP) produces a fragment showing complete immunologic identity to vWF:Ag.[10]

This paper presents evidence that polymorphonuclear (PMN) cells also contain an enzyme(s) that proteolytically cleaves vWF to produce a fragment that is immunologically related to vWF:Ag. This enzyme has different characteristics from the platelet CAP recently reported to cleave vWF:Ag.[10]

MATERIALS AND METHODS

Chemicals were purchased from the following suppliers: agarose (Seakem, Marine Colloids Division, Maine); barbital (sodium salt), soybean trypsin inhibitor (SBTI), phenylmethyl sulfonyl fluoride (PMSF), hirudin, leupeptin, benzamidine, sodium diatrizoate, and diisopropyl fluorophosphate (DFP) (Sigma Chemical Co, St Louis); sodium heparin (Weddell Pharmaceuticals, Wrexham, UK); aprotinin (Bayer Pharmaceuticals, Sydney, Australia); sodium dodecyl sulfate (SDS) (Pierce Chemical Co, Rockford, Ill); sucrose (Ajax Chemicals, Melbourne, Australia); Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden); urea (BDH Chemicals, Melbourne, Australia); epicron (EACA) (Difrex, Sydney, Australia); and calcium ionophore A23187 (Calbiochem-Behring Corp, La Jolla, Calif). Ristocetin was kindly supplied by Lundbeck & Co (Copenhagen) and dissolved in 0.15 mol/L saline at a concentration of 20 mg/mL.

Plasma and serum samples. Whole blood was collected into a 1/10 volume of 3.8% trisodium citrate. Platelet-poor plasma (PPP) was obtained by centrifugation at 2,000 g for 20 minutes at room temperature (RT). Normal pooled plasma (NPP) from 20 normal donors was prepared from citrated blood by centrifugation at 17,000 g for 20 minutes at 4 °C. For serum preparation, whole blood was collected into polystyrene tubes, allowed to clot at 37 °C for two hours, and left overnight at 4 °C. The serum was obtained by centrifugation at 2,000 g for 20 minutes at RT.

Cell preparation. Buffy coats were prepared from heparinized whole blood by centrifugation at 2,000 g for 15 minutes. The leukocyte-platelet layer was extracted and diluted in 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl buffer containing 5 mmol/L glucose and 6 mmol/L potassium EDTA, pH 7.4. The human mononuclear (MN) and PMN leukocytes were separated by centrifugation on a Ficoll-Hypaque medium with a density of 1.095 g/mL as described.[11]

Platelet contamination was reduced in the leukocyte preparations by centrifugation on a discontinuous sucrose gradient (6%, 12%, and 16% sucrose) as described by Perper et al.[12] Platelets form a layer in the 4% to 8% sucrose zone and were easily separated from the leukocytes, which sedimented through the entire sucrose gradient. All cell preparations (PMN, MN, platelets, and erythrocytes [red blood cells (RBC) obtained from the initial centrifugation step]) were washed three times with 0.02 mol/L Tris-HCl, 0.15 mol/L NaCl buffer containing 5 mmol/L glucose, and 6 mmol/L potassium EDTA, pH 7.4. Following the washing procedure, the cells were resuspended in the washing buffer.

Table 1 shows the distribution of cells present in the various cell preparations from one representative experiment.

Purification of RCof/vWF:Ag. Semipurified antihaemophiliac factor (Commonwealth Serum Laboratories (CSL), Melbourne)

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Table 1. Distribution of Cells Present in the Isolated Cell Preparations

Cell Preparation	Differential Count (%)		Cell Count (× 10^{11}/L)	
	MNC	PMNC	WBC	Platelets
MNC	99	1	1.5	2.88
PMNC	11.5	88.5	1.2	1.48
Platelets	ND	ND	0.48	162.0
RBC	ND	ND	0.07	ND

Abbreviation: ND, not determined.

was dissolved in 25% sucrose in 50 mmol/L Tris and 0.15 mmol/L NaCl containing 10 U/mL aprotinin and 0.02% sodium azide and subjected to centrifugation at 100,000 g at 20 °C for 30 minutes (to exclude lipids and particulate matter). A 2-mL sample of the infranatant was then applied to a Sepharose 4B-Cl column (3 cm × 80 cm), and 2.8-mL fractions were collected. Early eluting fractions of RCof/vWF:Ag free from detectable fragments of vWF:Ag were pooled and used as a source of purified RCof/vWF:Ag.

vWF analysis. RCof activity was measured as previously described[13] using formaldehyde-fixed platelets. vWF:Ag was measured by electroimmunoassay (EIA).[14] Radiocrossed immunoelectrophoresis (radio-CIE) in agarose was performed as described.[9,15] A 50-μL sample was electrophoresed toward the anode in the first dimension until an Evan's blue marker migrated 7 cm from the origin. In the second dimension the sample was electrophoresed into agarose containing a mixture of whole rabbit antiserum to RCof/vWF:Ag and immunoaffinity purified, radiolabeled rabbit IgG to RCof/vWF:Ag. At the end of the electrophoresis, the plates were washed and dried, and autoradiography was allowed to proceed at −70 °C for up to 120 hours, depending on the batch of ^{125}I-labeled anti-RCof/vWF:Ag IgG.

vWF:Ag multimeric analysis. Purified vWF was labeled with ^{125}I by the iodogen method (as described by the manufacturer, Pierce Chemical Co, Rockford, Ill). The multimeric composition of ^{125}I-vWF:Ag following incubation with the lysed PMNC preparation was visualized after SDS–agarose gel electrophoresis.

Slab gels (10 cm × 7 cm) containing 6 mol/L urea, 0.2% SDS, and 1% agarose in 0.075 mmol/L barbital buffer, pH 8.6, were prepared in glass molds and set at 4 °C for one hour. The gels were then transferred onto gelbond (FMC Corp, Marine Colloids Division, Rockland, Maine), and five wells (0.4 cm × 1.0 cm) were cut out, ready for use. The tank buffer was 0.075 mmol/L barbital buffer, pH 8.6, containing 0.2% SDS. In a typical experiment, 30 μL ^{125}I-vWF:Ag (0.5 U/mL) was added to 30 μL of a lysed PMNC suspension (1.4 × 10^8 mL) or 30 μL Tris/saline buffer (control) and incubated at 37 °C for up to 60 minutes. Each sample was diluted 1/5 in gel buffer (see the preceding material) and 40 μL applied to wells in a SDS/urea/agarose gel and left to set before electrophoresis at 12 mA for four hours. The gels were then dried, stained, and autoradiographed.

RESULTS

Fragments of vWF:Ag in blood products. Examination of radio-CIEs of NPP, normal serum, plasma from a patient with DIC, and a factor VIII concentrate (CSL) show the presence of an additional vWF:Ag precipitin peak (Fig 1). Figure 1 (B, C, and D) shows the presence of a precipitin peak, (indicated by the arrows) previously called VIII:FMP[9] (fast-migrating protein). This vWF:Ag fragment is clearly visible in the radio-CIEs of DIC plasma and factor VIII concentrate, is present in smaller amounts in normal serum, and is faintly detectable in normal plasma. This antigen shows partial immunologic identity to the major vWF:Ag peak. A spur to the main vWF:Ag precipitin peak can be seen in Fig 1C (denoted by P), suggesting the presence of additional vWF:Ag fragments.

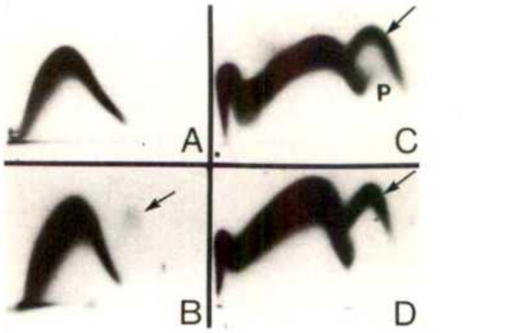

Fig 1. Radio-CIE patterns of blood products. (A) NPP. (B) Normal serum. (C) Plasma from a patient with DIC. (D) CSL antihaemophiliac factor concentrate. The arrow indicates FVIII:FMP. A spur reaction on the vWF:Ag precipitin peak is shown in (C) and indicated by P.

Effect of lysed purified cell preparations on purified RCof/vWF:Ag. PMNC, MNC, RBC, and platelets at comparable cell concentrations in Tris, saline, glucose, and EDTA buffer, pH 7.4, were lysed by rapid freeze-thawing four times. Each preparation was incubated with purified RCof/vWF:Ag (0.25 U/ML, final concentration) for 60 minutes at 37 °C.

Figure 2 shows the radio-CIE of the resultant incubation mixtures. No vWF:Ag fragments were detected in the purified RCof/vWF:Ag preparation or were produced during a 60-minute incubation at 37 °C with buffer, RBC, MNC, or platelets. Incubation with PMNC generated vWF:Ag fragments (Fig 2E) during the 60-minute incubation at 37 °C. One of these fragments (indicated by the arrow) shows partial immunologic identity to the main vWF:Ag precipitin peak, and a second spur (labeled P) shows greater immuno-

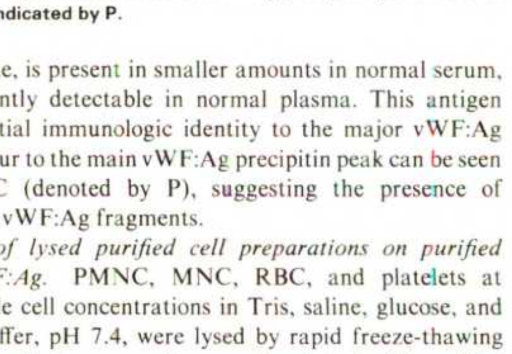

Fig 2. Radio-CIE patterns of purified RCof/vWF:Ag (0.25 U/mL final concentration) incubated for 60 minutes at 37 °C with (A) buffer, (B) lysed RBC (concentration not determined), (C) lysed platelets (8.1 × 10^7 mL), (D) lysed MNC (0.75 × 10^8 mL), (E) lysed PMNC (0.50 × 10^8 mL), and (F) lysed PMNC (0.50 × 10^8 mL) incubated with buffer in the absence of RCof/vWF:Ag. All concentrations are their final concentration in the incubation mixture. This experiment was repeated twice with similar results.

logic similarity to vWF:Ag. Fragmentation of vWF:Ag was still detected if the lysed PMNC preparation was incubated with purified RCof/vWF:Ag at a final cell concentration of 1.25 × 10⁷ mL (data not shown).

Time course of RCof/vWF:Ag fragmentation by lysed PMNC. The RCof activity of a purified RCof/vWF:Ag preparation was progressively lost during a 20-minute incubation at 37 °C with lysed PMNC, whereas the vWF:Ag level decreased only slightly during a 60-minute incubation period (Fig 3). In the absence of cells, a small but insignificant fall in both RCof activity and vWF:Ag levels was observed. A progressive increase in the height and density of the vWF:Ag fragment peak was seen on the resultant radio-CIE autoradiograms (Fig 4) of aliquots taken from the incubation mixture at intervals during the 60-minute incubation described in Fig 3. It may also be noted from Fig 4 that as the incubation proceeded and the fragment of vWF:Ag was produced a loss of the high–molecular weight forms of vWF:Ag occurred (Fig 4E).

The loss of high–molecular weight multimers of vWF:Ag was further demonstrated by SDS-agarose electrophoresis (Fig 5). Purified vWF:Ag was radiolabeled with $^{125}I_2$ and incubated with PMNC for 60 minutes. Aliquots were removed from the incubation mixture at 5, 15, 30, and 60 minutes and subjected to SDS-agarose electrophoresis. A typical autoradiograph obtained from one of these experiments clearly demonstrated a progressive loss of high–molecular weight multimers and a simultaneous increase in smaller–molecular weight forms of vWF:Ag (Fig 5). The arrowhead in Fig 5 indicates the presence of multimeric forms of vWF:Ag (lanes 3 to 5) that are not present in the starting material (lane 1). These multimers would appear to correspond to the additional precipitin peaks seen on radio-CIE of similar incubation mixtures. Extension of the incubation period to four hours at 37 °C resulted in a reduction in the level of both vWF:Ag and the fragment of vWF:Ag (data not shown).

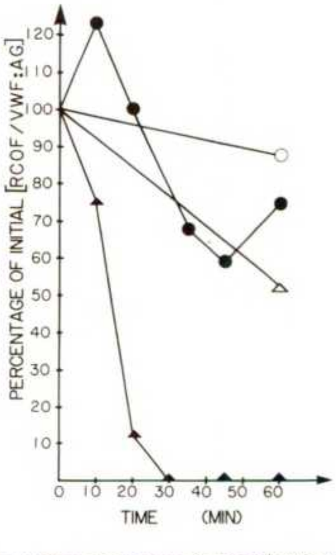

Fig 3. Time course of changes in RCof/vWF:Ag levels during incubation at 37 °C. Purified RCof/vWF:Ag (0.25 U/mL, final concentrations) was incubated with buffer for 0 to 60 minutes (△, RCof activity; ○, vWF:Ag). Purified RCoF/vWF:Ag (0.25 U/mL, final concentration) was incubated with lysed PMNC (0.5 × 10⁸/mL, final concentration) for 0 to 60 minutes. ▲ (RCof) and ● (vWF:Ag) were measured at various intervals during the 60-minute incubation. Similar results were obtained in an identical experiment.

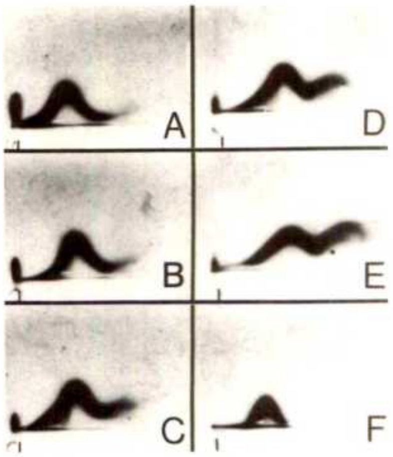

Fig 4. Radio-CIE patterns from the time course experiment (Fig 3). Purified RCoF/vWF:Ag (0.25 U/mL, final concentration) was incubated with lysed PMNC (0.5 × 10⁸/mL, final concentration) for (A) 0 minutes, (B) 10 minutes, (C) 30 minutes, (D) 45 minutes, (E) 60 minutes, and (F) purified RCof/vWF:Ag incubated with buffer for 60 minutes. Similar results were obtained in an identical experiment.

Effect of temperature on RCof/vWF:Ag fragmentation. The temperature sensitivity of the PMNC protease responsible for RCof/vWF:Ag fragmentation was investigated by incubating purified RCof/vWF:Ag (0.29 U/mL, final concentration) and buffer or PMNC (0.5 × 10⁸ mL, final concentration) for 60 minutes at 4 °C, 20 °C, 37 °C, 45 °C, and 56 °C.

Figure 6A shows that, in the presence of buffer, vWF:Ag and RCof activity were stable to temperatures of 45 °C for 60 minutes but RCof activity was greatly reduced above this temperature. No significant change in the radio-CIE pattern of vWF:Ag was apparent for the temperature range of 4 °C to 56 °C following a 60-minute incubation.

Incubation of the purified RCof/vWF:Ag (0.29 U/mL, final concentration) with PMNC (0.5 × 10⁸/mL, final con-

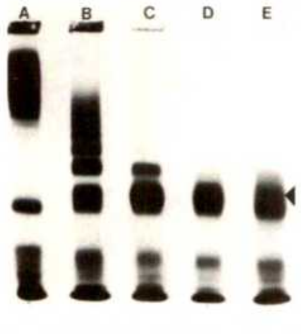

Fig 5. SDS-agarose gel electrophoresis: ^{126}I-purified RCof/vWF:Ag (0.5 U/mL) incubated with buffer for 60 minutes (A), purified RCof/vWFAg incubated with lysed PMNC (1.4 × 10⁸/mL) for 5 minutes (B), 15 minutes (C), 30 minutes (D), and 60 minutes (E). Similar results were obtained in six identical experiments.

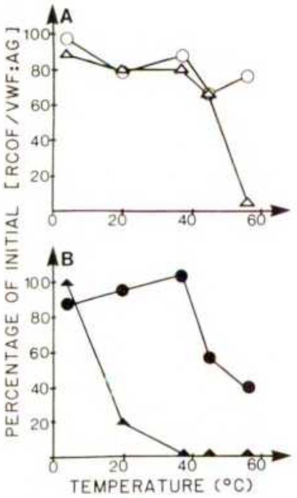

Fig 6. (A) Purified RCof/vWF:Ag (0.29 U/mL) was incubated with buffer at various temperatures from 4 °C to 56 °C for 60 minutes. △, RCof; ○, vWF:Ag. (B) Purified RCof/vWF:Ag (0.29 U/mL) was incubated with lysed PMNC (0.5 × 10^8/mL) at 4 °C to 56 °C for 60 minutes. ▲, RCof; ●, vWF:Ag. Similar results were obtained from two duplicate experiments.

centration) for 60 minutes caused a significant decrease in RCof activity at 20 °C and complete inactivation of RCof at temperatures of 37 °C to 56 °C. In the presence of PMNC, the level of vWF:Ag was unaffected until the temperature was > 37 °C (Fig 6B) when a progressive decrease in its level occurred as the temperature of incubation increased. The CIE patterns for vWF:Ag in the presence of PMNC are shown in Fig 7. No fragmentation of vWF:Ag was detected by radio-CIE following incubation of RCof/vWF:Ag with PMNC at 4 °C or 20 °C (Fig 7A). Incubation at 37 °C resulted in vWF:Ag fragment production (Fig 7B), which was also present at higher incubation temperatures (Fig 7C).

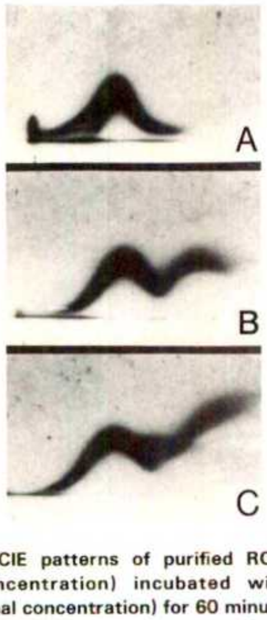

Fig 7. Radio-CIE patterns of purified RCof/vWF:Ag (0.29 U/mL, final concentration) incubated with lysed PMNC (0.5 × 10^8/mL, final concentration) for 60 minutes at (A) 4 °C, (B) 37 °C, and (C) 45 °C. Radio-CIE from two identical experiments yielded similar patterns.

Effect of activation of intact PMNC. Fragments of vWF:Ag were not detected by radio-CIE following incubation of purified RCof/vWF:Ag with fresh intact PMNC (Fig 8A).

Inclusion of 0.2 mmol/L $CaCl_2$ or 0.17 mmol/L calcium ionophore (Fig 8B, 8D) in the incubation mixture of purified RCof/vWF:Ag with intact PMNC resulted in production of vWF:Ag fragments following 60 minutes at 37 °C.

Effect of inhibitors on RCof/vWF:Ag degradation by PMNC proteases. Fragmentation of vWF:Ag by PMNC was inhibited by adding either SBTI (33 mg/mL), DFP (0.6 mmol/L), or Trasylol (1,500 kIU/mL) to RCof/vWF:Ag immediately before incubation with lysed PMNC for 60 minutes. Other inhibitors—leupeptin (1.3 mmol/L), benzamidine (15 mmol/L), sodium azide (3 mmol/L), EACA (1.5 mol/L), aprotinin (150 kIU/mL), hirudin (1.0 U/mL), EDTA (5.0 mmol/L), and trisodium citrate (13 mmol/L)—did not inhibit the action of PMNC proteases on purified RCof/vWF:Ag. At the concentrations reported, none of these agents had any effect on the radio-CIE pattern of RCof/vWF:Ag.

DISCUSSION

In vivo fragmentation of the RCof/vWF:Ag molecule has been described in association with events known to cause activation of proteolytic enzymes such as DIC,[7-9] urokinase therapy,[7] and sickle cell crisis.[16] The actual fragment of vWF:Ag demonstrated by radio-CIE appears to vary. This may depend to some extent on the antibody used in the second dimension of the radio-CIE. Using a rabbit antibody raised against vWF:Ag, we were able to demonstrate the presence of fragmentation products previously termed VIII:FMP and VIII:P[9] in the plasma from patients with DIC and by in vitro manipulation of purified vWF:Ag (Figs 1 and 2). These products would appear to correspond to the VIII:Ag:fragment described by Montgomery and Johnson[8] and fast-moving peak described by Lombardi et al.[7]

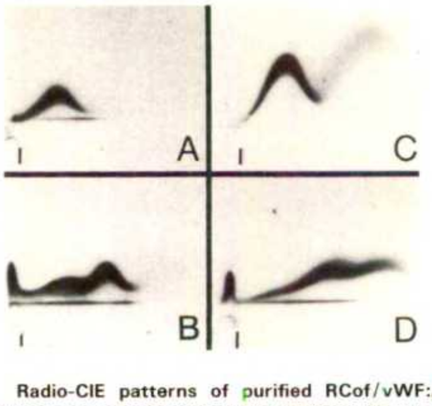

Fig 8. Radio-CIE patterns of purified RCof/vWF:Ag (0.60 U/mL, final concentration) incubated at 37 °C for 60 minutes with (A) intact PMNC (0.9 × 10^8/mL, final concentration), (B) intact PMNC + calcium ionophore (0.17 mol/L, final concentration), (C) lysed PMNC (0.5 × 10^8/mL, final concentration), and (D) intact PMNC + $CaCl_2$ (0.2 mol/L, final concentration). Similar results were obtained in a duplicate experiment.

Experiments reported here demonstrate that PMNC contain an enzyme(s) that when released (Figs 2, 4, 6, and 7) and incubated with purified RCof/vWF:Ag results in the appearance of an additional vWF:Ag peak on radio-CIE. The relationship between this product and the formerly reported VIII:FMP or VIII:P is at present not proven.[9] However, electrophoretic mobilities on radio-CIE indicate a similar migration distance for the PMNC-produced RCof/vWF:Ag fragment and those present in the plasma from patients with DIC and in serum formed by allowing whole blood to clot in glass tubes.

The PMNC enzyme(s) responsible for RCof/vWF:Ag fragmentation is located intracellularly. Release of the enzyme(s) was achieved by freeze-thaw lysis of the cells or by cell activation. Suitable activators were calcium or the calcium ionophore A23187. The enzyme(s)' activity is inhibited by DFP, SBTI, and high concentrations of aprotinin, but not by benzamidine, azide, EACA, or hirudin. Addition of citrate, EDTA, or leupeptin had no effect on the activity of the enzyme(s), indicating that the PMNC enzyme(s) investigated in these experiments is not calcium dependent, although calcium is able to activate PMNC to release the enzyme(s). The possibility exists that PMNC also contain CAP similar to those demonstrated in platelets that have been shown to cleave vWF:Ag.[10] The design of the experiments reported here would have precluded demonstration of action by such enzymes since all cells were washed in a buffer containing 6 mmol/L EDTA, a concentration high enough to inactivate the platelet CAP.[10]

The experiments comparing the temperature dependence of loss of RCof activity and vWF:Ag degradation showed a concomitant loss of RCof activity and appearance of fragments of vWF:Ag after incubation with PMNC at temperatures between 37 °C and 56 °C.

The concentration of lysed PMNC used to obtain fragmentation of vWF:Ag is more than 2.5 times that found in blood. During clot formation, PMNC cluster around the platelet/fibrin mass, and so it is not inconceivable that a local increase in PMNC concentration could occur. Using PMNC elastase as a marker for PMN leukocyte activation, Plow[17] reported release of elastase during serum production. Further evidence of release of leukocyte constituents during coagulation is indicated by the higher lactoferrin levels found in serum than plasma in a study by Bennett and Mohla.[18] Thus, liberation of PMNC constituents during clot formation is indicated, and the in vitro fragmentation of vWF:Ag demonstrated with lysed or calcium-activated PMNC may parallel in vivo events.

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REFERENCES

1. Guisasola JA, Cockburn CG, Hardisty RM: Plasmin digestion of factor VIII: Characterization of the breakdown products with respect to antigenicity and von Willebrand activity. Thromb Haemost 40:302, 1978

2. Kirby EP, Martin N, Marder VJ: Degradation of bovine factor VIII by plasmin and trypsin. Blood 43:629, 1974

3. Atichartakarn V, Marder VJ, Kirby EP, Budzynski AZ: Effects of enzymatic degradation on the subunit composition and biological properties of human factor VIII. Blood 51:281, 1978

4. Kopec M, Bykowska K, Lopacink S, Jelenska M, Kaczanowska J, Sopata I, Wojtecka E: Effects of neutral proteases from human leukocytes on structure and biological properties of human factor VIII. Thromb Haemost 43:211, 1980

5. Rick ME, Hoyer LW: Thrombin activation of factor VIII: The effect of inhibitors. Br J Haematol 36:585, 1977

6. Marlar RA, Kleiss AJ, Griffin JH: Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. Blood 59:1067, 1982

7. Lombardi R, Mannucci PM, Seghatchian MJ, Garcia VV, Coppola R: Alterations of factor VIII von Willebrand factor in clinical conditions associated with an increase in its plasma concentration. Br J Haematol 49:61, 1981

8. Montgomery RR, Johnson J: Specific FVIII-related antigen fragmentation: An in vivo and in vitro phenomenon. Blood 60:930, 1982

9. Thomas KB, Howard MA, Salem HH, Firkin BG: Fast migrating protein, immunochemically related to human factor VIII, studied by crossed immunoelectrophoresis in agarose. Br J Haematol 54:221, 1983

10. Kunicki TJ, Montgomery RR, Schullek J: Cleavage of human von Willebrand factor by platelet calcium-activated protease. Blood 65:352, 1985

11. Ferrante A, Thong Y: Simultaneous preparation of mononuclear and polymorphonuclear leucocytes from horse blood on Ficoll-Hypaque medium. J Immunol Methods 34:279, 1980

12. Perper RJ, Zee TW, Mickelson MM: Purification of lymphocytes and platelets by gradient centrifugation. J Lab Clin Med 72:842, 1968

13. MacFarlane DE, Stibbe J, Kirby EP, Zucker MB, Grant R, McPherson J: A method for assaying von Willebrand factor (ristocetin cofactor). Thombosis Diathesis Haemorrhagica 34:306, 1975

14. Laurell CB: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Ann Biochem 15:45, 1966

15. Koutts J, Walsh PN, Plow EF, Fernon JW, Bouma BN, Zimmerman TS: Active release of human platelet factor VIII-related antigen by adenosine diphosphate, collagen and thrombin. J Clin Invest 62:1255, 1978

16. Mackie I, Bull H, Brozovic M: Altered factor VIII complexes in sickle cell disease. Br J Haematol 46:499, 1980

17. Plow EF: Leukocyte elastase release during blood coagulation. J Clin Invest 69:564, 1982

18. Bennett RM, Mohla C: A solid-phase radioimmunoassay for the measurement of lactoferrin in human plasma. J Lab Clin Med 88:156, 1976

Separation of Human Megakaryocytes by State of Differentiation on Continuous Gradients of Percoll: Size and Ploidy Analysis of Cells Identified by Monoclonal Antibody to Glycoprotein IIb/IIIa

By Toshiyuki Ishibashi, Zaverio M. Ruggeri, Laurence A. Harker, and Samuel A. Burstein

Human bone marrow was separated on continuous Percoll density gradients to analyze the distribution of cells of the megakaryocytic lineage. Megakaryocytes were identified by indirect immunofluorescence using a monoclonal antibody (LJP4) specific to the glycoprotein IIb/IIIa (GPIIb/IIIa) complex of platelets. Neither endothelial cells nor monocytes expressed the epitope identified by this antibody. Simultaneous measurement of size and ploidy were made on 2,359 GPIIb/IIIa-positive cells. The smallest cells were located in the most dense fractions where 81% of all 2N and 66% of 4N cells were found at densities $\geq$1.050 g/mL. The largest cells were detected in the least dense fractions, with 70% of 16N, 78% of 32N, and 100% of 64N cells found at densities $<$1.050 g/mL. Ninety-four percent of all GPIIb/IIIa-positive cells $<$14 μm in diameter were found at densities $>$1.050 g/mL. An exception to this inverse relationship was observed in the uppermost gradient fractions where an anomalous distribution of size and ploidy was found. Megakaryocytic viability was identified as being greater than 90% in all fractions. The data show that megakaryocytic differentiation as assessed by size and ploidy varies inversely with Percoll density. Separation of marrow on continuous Percoll gradients may be a useful method to separate megakaryocytes at different stages of differentiation.

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THROUGHOUT the past decade technical advances in separation technology and in vitro assay methods have led to new insights into the structure and regulation of the megakaryocytic lineage in animals.[1-5] It has only been in the past several years, however, that similar technology has been available for the study of human megakaryocytopoiesis. These technical advances have included in vitro semisolid assays for the human megakaryocytic colony-forming cell,[6-9] identification of immature and mature megakaryocytes by means of specific antisera,[10-17] and separation techniques to enrich for megakaryocytes.[10,18,19]

In mice, the relatively specific marker enzyme acetylcholinesterase has been used to identify early cells of the megakaryocytic lineage that would be otherwise unrecognizable.[20,21] In a recent study using this enzyme marker, we examined the use of continuous Percoll gradients to separate murine megakaryocytes and found that megakaryocytic differentiation as assessed by cell size and ploidy was inversely proportional to density.[22] Since human megakaryocytes are enriched at relatively low density on Percoll gradients whereas megakaryocytic progenitor cells are enriched at higher density, it seems likely that human megakaryocytic differentiation is also inversely related to Percoll density.[9,10] In this study, we have separated human marrow on continuous Percoll gradients using a monoclonal antibody to the platelet-specific glycoprotein IIb/IIIa (GPIIb/IIIa) complex to identify megakaryocytes.[13,14] The data show that low-ploidy cells express GPIIb/IIIa and that human megakaryocytes separate according to their state of differentiation on Percoll gradients.

MATERIALS AND METHODS

Marrow cells. Human bone marrow was aspirated from the posterior iliac crest of normal volunteer donors. Marrow was obtained after informed consent according to the guidelines of the Human Subjects Committee of Scripps Clinic and Research Foundation. The marrow was collected into sterile syringes containing preservative-free heparin (50 U/mL; Sigma Chemical Company, St Louis, Mo). Within 20 minutes of aspiration, the marrow was diluted with 3 vol of suspension medium consisting of 0.129 mol/L NaCl, 8.614 mmol/L Na_2HPO_4, 1.6 mmol/L KH_2PO_4, 13.6 mmol/L Na citrate, 11.1 mmol/L glucose, 1 mmol/L adenosine, 2 mmol/L theophylline, 2.3×10^{-6} mol/L prostaglandin E_1 (PGE_1), and 118.4 KU/mL of DNAase I (Calbiochem-Behring Corp, La Jolla, Calif). The pH of the media was adjusted to 7.0, and the osmolarity was adjusted to 295 ± 3 mosm/L. The marrow was gently pipetted with a siliconized Pasteur pipette.

Separation of marrow cells. The cell suspensions were first layered over a single-density cut of Percoll at 1.080 g/mL. Percoll was diluted to the desired density with suspension medium. No more than 6 mL of cell suspension was layered over 6 mL of Percoll in 15-mL tissue culture tubes. After centrifugation at 400 g for 20 minutes at room temperature, the cells were collected, washed once in suspension medium, and resuspended into 10 mL of the same medium. A 40-mL 30% to 63% continuous Percoll density gradient was formed with a gradient maker (Buchler Density Gradient System, Haake Buchler Instruments Inc, Saddlebrook, NJ) and layered into a 50-ml plastic culture tube (Corning Glass Works, Corning, NY) at a rate of 2.5 mL/min. Ten milliliters of the marrow cell suspension were layered over the gradient, and the cells were centrifuged at 400 g for 20 minutes at room temperature. Subsequently, the bottom of the tube was punctured with an 18-gauge needle, and the gradient was separated into 25 2-ml fractions at 2.5 mL/min using a peristaltic pump and fraction collector (PI and FRAC 100, Pharmacia Fine Chemicals, Piscataway, NJ).

Cell counts and viability. Cell counts were performed with an automated cell counter (Hematology Series 150, Baker Instrument Co, Allentown, Pa) both before and after washing the cells in each fraction. Total cellular viability was determined by trypan blue exclusion and acridine orange inclusion–ethidium bromide exclusion in each fraction.[23]

Megakaryocytic viability was specifically ascertained by the

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fluorescein diacetate (Sigma) method.[24,25] In this technique, viable cells incorporate fluorescein diacetate and metabolize the dye, resulting in intense intracellular fluorescence, whereas nonviable cells do not. Cells from each fraction were first incubated with monoclonal antibody LJP4 for 30 minutes at 0 °C (12.6 μg/mL of the purified IgG diluted in 0.1% ovalbumin). Subsequently, the cells were washed and then incubated with a 1:20 dilution (in 0.1% ovalbumin) of rhodamine-conjugated goat $F(ab')_2$ antimouse IgG (Tago, Inc, Burlingame, Calif) and a fluorescein diacetate solution (0.1 μg/mL) for 20 minutes at 0 °C. After washing, the cells of each fraction were examined with a Zeiss Photoscope 3 (Carl Zeiss, Oberkochen, West Germany) equipped for both fluorescein and rhodamine fluorescence. Megakaryocytes were first identified by using the rhodamine filters; subsequently, the fluorescein filters were used to assess viability. Living cells exhibited bright green fluorescence.

Studies with a monoclonal antibody to monocytes. To determine whether platelet fragments adhere to the surface of monocytes and result in the misidentification of such cells as megakaryocytes, double fluorescence-labeling studies were performed. A 1:100 dilution of both anti–Mac-1 (Hybritech, San Diego), a rat antimouse monoclonal antibody with cross-reactivity to human monocytes and natural killer cells,[26] and LJP4 were applied to separated marrow cells for 30 minutes, washed, and followed by the simultaneous 30-minute incubation with a 1:32 dilution of fluoresceinated goat antirat IgG and rhodamine goat antimouse IgG as previously described. After washing, slides were examined for expression of both antigens.

Studies with endothelial cells. To determine whether human endothelial cells react with a monoclonal antibody to GPIIb/IIIa, one-day-old primary cultures of human umbilical vein endothelial cells grown on glass coverslips were incubated with LJP4 at 12.6, 63, and 126 μg IgG/mL for two hours. After rinsing with phosphate-buffered saline (PBS) the fluoresceinated $F(ab')_2$ fragment of goat antimouse IgG was added for 30 minutes. The coverslips were rinsed and examined for fluorescence. As a positive control, some coverslips were treated with monoclonal antihuman von Willebrand factor (kindly provided by Dr T. Zimmerman, Scripps Clinic and Research Foundation). The expected granular fluorescence pattern was observed.[27]

Studies with a monoclonal antibody to GPIb. To determine whether cells positive for GPIIb/IIIa also express GPIb, 59 GPIIb/IIIa-positive cells identified by rhodamine fluorescence were mapped by recording the microscope stage coordinates of their location. The slides were then incubated for three hours at room temperature with biotinylated LJP3 IgG, a monoclonal antibody produced as will be described. After rinsing with PBS, the slides were incubated with an avidin-biotin-glucose oxidase complex (Vectastain; Vector Laboratories, Burlingame, Calif) for one hour, rinsed, and incubated with the glucose oxidase substrate for 20 minutes. Positive cells were identified by a purple deposit.

Monoclonal antibody preparation. Monoclonal antibodies against human platelet membrane glycoproteins were produced by immunizing BALB/c mice with intraperitoneal injections of washed platelet suspensions. Platelets were prepared by gel filtration of platelet-rich plasma through Sepharose CL 2B (Pharmacia) as previously described with the omission of albumin.[28] The first injection consisted of 10^8 platelets in complete Freund's adjuvant. Two boosters of 10^8 and 1.5×10^8 platelets, respectively, in incomplete Freund's adjuvant were given at 1-week intervals. A final booster of 3×10^8 platelets without adjuvant was given four days before fusion. Mouse spleen cells were fused with mouse plasmacytoma cells at a ratio of 6:1. The antibody used for the present study, designated LJP4, is an IgG_1 and was derived from a fusion with P3X63–Ag 8.653 plasmacytoma cells. Fusion and growth of hybridomas were performed using standard procedures.[29] Positive clones producing antiplatelet antibodies were selected by enzyme-linked immunoadsorbent assay.[30] This assay detected mouse IgG reacting with washed platelets bound to plastic microtiter wells by means of poly-L-lysine hydrobromide having a molecular weight of 150,000 to 300,000 (Sigma). Positive clones were subcloned twice by limiting-cell dilution.[31] Monoclonal antibody was then produced in mouse ascites fluid as described.[29]

Purified IgG was obtained from ascites fluid by binding to protein A–Sepharose (Sigma) followed by stepwise elution at pH 6, 4.5, and 3.[32] Subclass specificity of the IgG molecules was defined by Ouchterlony immunodiffusion against the appropriate antisera.[33] LJP4 bound to the GPIIb/IIIa heterodimer complex, but not to isolated GPIIb or GPIIIa following dissociation of the complex by treatment with EDTA at pH 8 at 37 °C for 15 minutes. This was determined by measuring both the binding of ^{125}I-labeled LJP4 IgG to whole platelets and the coprecipitation of the labeled antibody with precipitin arcs formed by membrane glycoproteins reacting with a polyclonal antiplatelet antiserum in a crossed immunoelectrophoretic system.[34]

Monoclonal antibody LJP3 IgG, specific to platelet GPIb, was prepared as previously described. One mg/mL of the IgG was dialyzed against 0.1 mol/L $NaHCO_3$ at 4 °C for six hours. After dialysis, 120 μL of biotin-N-hydroxysuccinimide ester (1 mg/mL in dimethyl sulfoxide) was added per milliliter of protein and incubated at room temperature for four hours. The biotinylated IgG was then dialyzed against PBS overnight.

Number, size, and ploidy measurements of megakaryocytes. Cytocentrifuge preparations of each fraction were performed using centrifuge buckets designed for quantitative purposes.[35] The cells were fixed with acetone and ethanol at a ratio of 3:17 for ten minutes at room temperature, washed with PBS, then rinsed with distilled water and dried. The specimens were first incubated with 12.6 μg/mL of the IgG fraction of monoclonal anti-GPIIb/IIIa antibody for 30 minutes at room temperature. Subsequently, the slides were washed three times with PBS, rinsed with distilled water, and dried. The slides were then incubated with a 1:48 dilution of rhodamine-conjugated goat $F(ab')_2$ antimouse IgG for 30 minutes at room temperature. Following washing three times in PBS and rinsing with distilled water, the slides were then stained with 1.7×10^{-5} mol/L chromomycin A3 (Calbiochem-Behring) for 30 minutes as described previously.[36] Cells in each microscopic field were examined under fluorescein, rhodamine, and phase-contrast optics. For identification of megakarocytes, rhodamine filters were used. Megakaryocytic diameters were established by measuring the geometric mean of two perpendicular diameters of each rhodamine-positive cell. Measurement of megakaryocytic ploidy was determined by fluorescence cytophotometry as described previously.[36] After identification of the megakaryocytic nature of the cell under the rhodamine filter combination, the filters were switched for measurement of ploidy by chromomycin A3 fluorescence emission. Granulocytes obtained from the pellet of the initial 1.080-g/mL density cut were used as the diploid standard. Cells were assigned to discrete ploidy classes by the method of Paulus et al.[37]

RESULTS

Density gradient conditions. The optimal density limits for maximal megakaryocytic separation and recovery was determined after preliminary experiments using initial Percoll concentrations ranging from 20% to 80%. The optimal density limits were ascertained to be 30% at the top and 63% at the bottom. The centrifugation time of 20 minutes was chosen after other experiments had shown that longer centrifugation times did not improve the separation. Similarly, no difference was observed in separation when centrifugation was performed at 4 °C rather than room temperature. The addition of marrow cells directly to the gradient during formation rather than pipetting the cell suspension on top of the gradient also did not affect the final separation.

The density of each fraction of the continuous Percoll gradient was determined directly by weighing identical volumes of the particular fraction and water. The density of each fraction is shown in Table 1.

Cell recovery. After a single density cut of 1.080 g/mL to rid the marrow sample of red cells and the majority of granulocytes, nucleated cell recovery was 64% ± 3% of the starting marrow cellularity. Final recovery after washing was 58% ± 5%. Megakaryocytic recovery was 82%. To ascertain the percentage of megakaryocytic cells that might have been lost in the pellet in the initial 1.080-g/mL Percoll density cut, 3×10^4 cells in this pellet were also examined for GPIIb/IIIa-positive cells. No positive cells were observed in four separate experiments. Occasional megakaryocytes were observed along the sides of the centrifuge tube. This together with disruption of some of these cells upon washing might account for the 18% loss.

Distribution of GPIIb/IIIa-positive cells. Megakaryocytes identified by indirect immunofluorescence against GPIIb/IIIa averaged 0.04% of the initial starting marrow cells. To determine whether monocytes might also express this antigen,[26] separated marrow was examined for the simultaneous presence of Mac-1 and GPIIb/IIIa. Of greater than 400 Mac-1–positive cells, none expressed GPIIb/IIIa. To preclude the possibility that endothelial cells might express epitopes of GPIIb/IIIa recognized by LJP4, human umbilical vein endothelial cells were examined by indirect immunofluorescence for LJF4 binding. No positive cells were found.

To assess whether GPIIb/IIIa-positive cells also expressed an additional platelet specific antigen, 59 GPIIb/IIIa-positive cells as assessed by rhodamine immunofluorescence were mapped as described and stained for the presence of GPIb by an avidin-biotin immunoenzymatic technique. Forty out of 43 (93%) cells greater than 20 μm in diameter, five out of seven (71%) cells less than 20 but greater than 14 μm in diameter and four out of nine (44%) cells less than 14 μm in diameter were positive for both GPIIb/IIIa and GPIb. Table 1 shows the distribution of glycoprotein-positive cells in each fraction. The number of megakaryocytes identified by monoclonal antibody was inversely proportional to the Percoll density with the exception of the uppermost two fractions. Sixty-two to 114 glycoprotein-positive cells per 10^4 cells were identified in fractions 18 through 24 where megakaryocytes were enriched to 29-fold. Figure 1 shows the density profile of nucleated marrow cells of density <1.080 g/mL together with the megakaryocyte profile. Sixty-one percent of all megakaryocytes are found at densities <1.050 g/mL (fractions ≥ 16), whereas only 6.2% of nucleated cells are found at these densities.

Table 1. Separation of Human Bone Marrow Cells on Continuous Percoll Gradients

Fraction	Density (g/mL)	Cells/Fraction* (%)	Viability† Total	Viability† MKs	Number of MKs‡	Enrichment of MKs
1	1.078	35 ± 4	100 ± 0.1	100 ± 0	0.3 ± 0.04	0.08
2	1.076	9 ± 2	100 ± 0	100 ± 0	0.7 ± 0.2	0.2
3	1.074	5 ± 1	100 ± 0	100 ± 0	0.8 ± 0.2	0.2
4	1.073	4 ± 1	100 ± 0	100 ± 0	1 ± 0.6	0.3
5	1.072	4 ± 1	100 ± 0.2	100 ± 0	1 ± 0.5	0.3
6	1.070	4 ± 1	100 ± 0.4	100 ± 0	2 ± 0	0.5
7	1.067	5 ± 1	100 ± 0.4	100 ± 0	3 ± 0.8	0.8
8	1.065	7 ± 3	100 ± 0	100 ± 0	4 ± 0.8	1
9	1.063	6 ± 2	100 ± 0.2	100 ± 0	4 ± 1	1
10	1.061	5 ± 1	100 ± 0.5	100 ± 0	5 ± 2	1
11	1.058	3 ± 1	100 ± 0.7	100 ± 0	5 ± 1	1
12	1.056	3 ± 1	100 ± 0.4	99 ± 1	13 ± 3	3
13	1.054	2 ± 0.6	100 ± 0.7	99 ± 1	17 ± 2	4
14	1.052	1 ± 0.6	99 ± 0.7	99 ± 2	21 ± 2	5
15	1.050	0.9 ± 0.4	99 ± 1	99 ± 0	24 ± 5	6
16	1.046	1 ± 0.5	99 ± 2	99 ± 0	31 ± 6	8
17	1.040	0.8 ± 0.2	98 ± 3	99 ± 1	46 ± 16	13
18	1.036	0.5 ± 0.2	96 ± 3	98 ± 2	62 ± 23	16
19	1.033	0.4 ± 0.2	96 ± 1	98 ± 1	74 ± 17	19
20	1.030	0.4 ± 0.2	94 ± 3	96 ± 2	101 ± 28	25
21	1.027	0.4 ± 0.2	90 ± 2	97 ± 2	114 ± 27	29
22	1.024	0.5 ± 0.2	87 ± 5	97 ± 2	104 ± 37	26
23	1.022	0.6 ± 0.2	83 ± 5	95 ± 1	108 ± 32	27
24	1.019	0.6 ± 0.2	87 ± 7	90 ± 3	80 ± 14	20
25	1.016	1 ± 0.6	95 ± 5	90 ± 2	30 ± 9	8

Abbreviation: MK, megakaryocyte.

*Percentage of total recovered cells in each fraction. The data represent the means ± 1 SD (eight experiments).

†Percentage of viable cells in each fraction.

‡Number of GPIIb/IIIa-positive cells (per 10^4 examined cells in the fraction; four experiments).

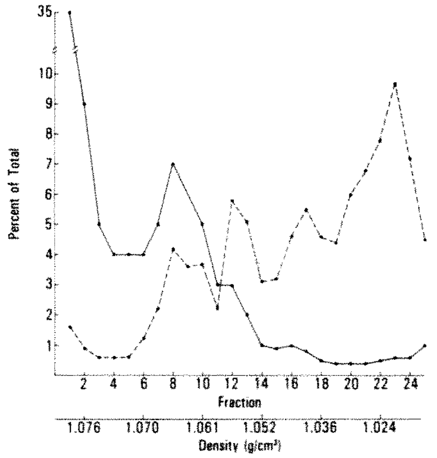

Fig 1. The density distribution of cells of density <1.080 g/mL separated on continuous Percoll gradients. The solid line represents the distribution of total nucleated cells whereas the broken line is the distribution of all GPIIb/IIIa-positive cells.

Viability. Cellular viability was determined by both trypan blue exclusion and acridine orange inclusion–ethidium bromide exclusion (Table 1). In comparison with trypan blue exclusion no significant difference was observed between the two methods except for several of the lower-density fractions in which a 5% to 11% increase in viability was observed with the latter fluorescent method (data not shown). Megakaryocytic viability was determined by simultaneous staining with fluorescein diacetate and indirect immunofluorescence against GPIIb/IIIa using a rhodamine label. Viability in all fractions was ≥90% (Table 1).

Megakaryocytic size. Geometric mean diameters were determined on 2,359 GPIIb/IIIa-positive cells and are shown in Table 2. The mean diameter of unfractionated bone marrow megakaryocytes was 34.1 ± 10.6 μm. The mean diameter of cells of individual fractions was inversely related to the Percoll density. The smallest mean diameters were observed in fractions 1 and 2 (15.3 ± 7.6 μm). The largest mean diameter was observed at fraction 22 (36 ± 8 μm). However, from fraction 23 through 25, an anomalous relationship was observed, with a direct relationship between size and apparent density.

If megakaryocytes are arbitrarily classified by size, 8% of all glycoprotein-positive cells are <14 μm in diameter, 8% are between 14 and 20 μm in diameter, whereas 84% are >20 μm in diameter. If one further classifies these cells according to whether they separate at > or <1.050 g/mL, 94% of all glycoprotein-positive cells <14 μm in diameter are found at densities >1.050 and 70% of intermediate-size cells are found at densities >1.050 g/mL, whereas only 31% of cells >20 μm in diameter are found at these densities. Figure 2 shows the density profiles of cells <14 μm in diameter and those >20 μm in diameter.

Ploidy. Ploidy measurements were obtained on the identical 2,359 cells on which size measurements were performed. The mean ploidy of each fraction is shown in Table 2, and is inversely related to the Percoll density between fractions 1 and 19, with a subsequent anomalous relationship noted in the least dense fractions similar to that observed with size. The lowest mean ploidy (3.9) was observed in fraction 1, whereas the highest mean ploidy (19.6) was noted in fraction 19. Table 3 shows the ploidy distribution of megakaryocytes in each fraction. Seventy percent to 80% of the megakaryocytes in the first six fractions were eiher 2N or 4N, with over half of the cells being 2N. A gradual decrease in the 2N and 4N cells is noted with progressively lower density, with these low ploidy cells becoming rare in fractions 15 and greater (1.050 g/mL). Cells of 8N and 16N are observed even at the highest densities, but are most frequent at lower density. The highest ploidy cells are observed in the least dense fractions, with rare 128N cells noted.

The ploidy frequency distribution of the normalized sum of all cells analyzed is also shown in Table 3. The predomi-

Table 2. Megakaryocyte Diameter and Ploidy of Separated Marrow

Fraction	Mean Diameter ± SD	Mean Ploidy ± SD	No. of Cells
Unfractionated	34.1 ± 10.6	16.9 ± 12.2	76
1	15.5 ± 5.9	3.9 ± 3.4	19
2	15.3 ± 7.6	4.4 ± 4.3	29
3	16.5 ± 5.7	4.4 ± 4.3	10
4	16.1 ± 9.1	4.4 ± 4.4	19
5	16.3 ± 9.3	4.6 ± 4.1	24
6	16.8 ± 9.4	5.3 ± 5.8	45
7	20.8 ± 11.1	7.3 ± 6.2	43
8	21.3 ± 10.1	7.0 ± 7.5	73
9	22.4 ± 10.0	8.6 ± 7.8	50
10	27.6 ± 9.9	10.6 ± 7.1	84
11	30.6 ± 10.7	12.6 ± 8.6	59
12	32.3 ± 9.5	12.8 ± 8.7	93
13	33.3 ± 8.5	14.1 ± 7.4	60
14	33.8 ± 9.1	14.5 ± 8.5	59
15	33.8 ± 8.3	16.1 ± 7.7	59
16	34.2 ± 6.1	16.8 ± 7.8	57
17	34.4 ± 8.1	17.2 ± 9.2	130
18	35.0 ± 7.5	17.7 ± 10.1	150
19	36.4 ± 7.9	19.6 ± 11.7	144
20	35.4 ± 6.9	19.3 ± 11.3	162
21	35.4 ± 7.2	18.6 ± 13.0	213
22	36.0 ± 8.0	17.7 ± 13.1	228
23	34.4 ± 7.5	17.2 ± 12.8	245
24	30.2 ± 8.6	10.9 ± 8.1	188
25	27.5 ± 11.0	10.4 ± 8.4	116

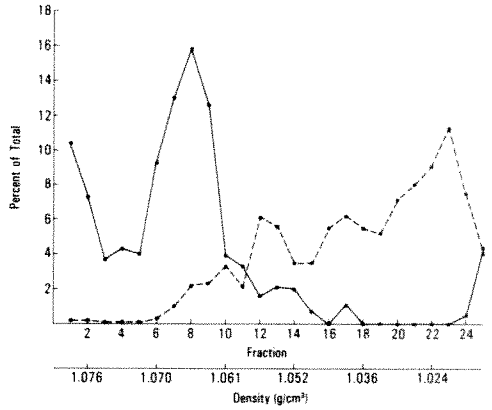

Fig 2. The density distribution of small (<14 μm, solid line) and large (>20 μm, broken line) GPIIb/IIIa-positive cells.

Table 3. Ploidy Distribution of Separated Megakaryocytes

Fraction	2N	4N	8N	16N	32N	64N	128N
Unfractionated	7*	8	21	42	20	2	
1	58	26	11	5			
2	62	18	10	10			
3	60	20	10	10			
4	62	16	11	11			
5	54	21	17	8			
6	55	16	18	9	2		
7	42	14	19	23	2		
8	26	25	33	15	1		
9	28	22	22	22	6		
10	10	14	40	30	6		
11	15	14	19	42	10		
12	9	7	39	32	13		
13	3	5	30	52	10		
14	5	5	32	43	15		
15	2	2	24	57	15		
16	0	2	23	58	17		
17	1	2	22	55	19	1	
18	0	1	23	57	17	2	
19	0	1	18	55	22	4	
20	1	1	23	47	26	3	
21	1	2	22	51	21	2	1
22	2	2	26	49	18	2	1
23	2	3	26	50	16	2	1
24	10	13	41	29	6	1	
25	14	17	39	21	9		
Total	10	8	27	41	13	1	

*Percentage of total.

nant ploidy class is 16N with 41% of the total cells. A significant number of the total cells were of low ploidy (10% 2N and 8% 4N). If one analyzes cells of various ploidies according to density, 81% of all 2N cells are found at densities >1.050 g/mL, whereas 66% of 4N cells are identified at these densities. The majority of higher-ploidy cells are found at densities <1.050 g/mL, with 60% of 8N, 70% of 16N, 78% of 32N, and 100% of 64N cells found at these low densities. Figure 3 shows the relationship between megakaryocyte ploidy and size of all the analyzed cells. The correlation between size and ploidy is fairly close, with a correlation coefficient of .762 ($P < .001$). Of interest is the size distribution of low-ploidy cells. The size distribution of 2N cells ranged from 7 to 46 μm with a mean of 13.3 ± 4.7 μm. The size of nonmegakaryocytes was 10.7 ± 2.8 μm (n = 702). Nineteen of 217 2N cells were between 20 and 30 μm, and one cell was 46 μm. The size range of 4N cells was 14 to 45 μm with a mean of 19.6 ± 5.2 μm. Seven of 166 4N cells were between 30 and 40 μm, and one cell was 45 μm.

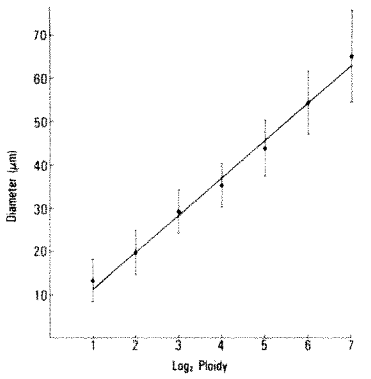

Fig 3. The relationship between mean cell diameter (closed circles) and ploidy. The abscissa represents discrete ploidy categories ($\log_2$) calculated according to the method of Paulus et al.[37] The error bars indicate ±1 SD of the mean. The line represents the computer-generated regression ($r = .762$, $P < .001$).

DISCUSSION

Previous studies have shown that megakaryocytes are enriched at low densities on Percoll gradients.[10] However, the megakaryocytic colony-forming cell is located at higher densities on Percoll (approximately 1.075 g/mL).[9] These findings suggest that as megakaryocytes mature from their progenitors, their apparent density on Percoll decreases. To test this hypothesis, we separated human marrow over continuous Percoll gradients and defined megakaryocytes as those cells expressing the platelet GPIIb/IIIa complex antigen using indirect immunofluorescence. Consequently, the megakaryocytic nature of the cells was established independently of size, ploidy, or morphology. Although GPIIb/IIIa has been reported to be present on monocytes in addition to platelets and megakaryocytes,[38,39] more recent studies have shown that the antigen is not intrinsic to monocytes.[40] However, the likelihood that the antigen is specific to the megakaryocytic lineage in aspirated marrow does not preclude the possibility that platelet fragments adhere to marrow monocytes[40] or that macrophages ingest effete megakaryocytes and transiently display antigens of that lineage. That no cells examined exhibited simultaneously both Mac-1 and GPIIb/IIIa mitigates against this possibility. Similarly, endothelial cells, a possible contaminant of marrow aspirates, were examined for expression of GPIIb/IIIa as defined by LJP4. Previous studies have suggested that endothelial cells express GPIIb and IIIa[41,42]; however, the present data suggest that not all epitopes of this glycoprotein complex are expressed by endothelial cells. Since it is possible that other monoclonal antibodies may identify such epitopes, endothelial cells should be prescreened with the antibody to rule out inadvertent misidentification.

Double-labeling studies with monoclonal antibody to GPIIb/IIIa and GPIb show that the vast majority of large megakaryocytes colabeled with both antibodies, whereas the smallest and presumably diploid cells labeled only with monoclonal antibody to IIb/IIIa. Studies by Vainchenker et al examining the time of expression of various platelet glycoproteins during in vitro culture have demonstrated that GPIIb/IIIa is the first platelet glycoprotein to be identified.[13] We conclude that the small cells expressing only GPIIb/IIIa are likely to be primitive cells in the megakaryocytic lineage, analogous to those found in day 7 in vitro colonies. Nevertheless, the full cellular range of expression of GPII/IIIa may as yet not be known. A recent report has suggested that monoclonal antibodies to GPIIb/IIIa may react in a cytotoxicity assay with murine nonmegakaryocytic stem cells.[43] It is thus conceivable that a population of nonmegakaryocytic cells may express the epitope of GPIIb/IIIa detected by LJP4. Despite these potential limitations, the use of the monoclonal antibody allows for high specificity with low background fluorescence and the ability to perform simultaneous size measurements and fluorescence cytophotometry for DNA content on the same cell. The data show that megakaryocytic differentiation is inversely related to the apparent density on Percoll.

The separation mode of megakaryocytes on Percoll differs from that on gradients of albumin, a phenomenon disparate from other cell types.[10,44] Rabellino has suggested that the differences in separation between Percoll and albumin may be due to the unique characteristics of the megakaryocytic membrane with its extensive demarcation membrane system.[45] Thus, the separation of megakaryocytes on Percoll may not necessarily be an accurate reflection of intrinsic megakaryocytic density. Nevertheless, this characteristic of megakaryocytes has made it possible to enrich megakaryocytes to virtual homogeneity using a single-density cut of Percoll as an initial separation modality.[10,19]

An exception to the inverse relationship between size or ploidy and apparent density is found in the upper several fractions, particularly fractions 24 and 25. In these fractions the mean diameter and ploidy are significantly less than several of the preceding fractions. This anomalous relationship was also observed in studies of murine megakaryocytes,[22] and its explanation is unclear. Dead cells are known to be enriched at low densities of Percoll, and it is conceivable that dead megakaryocytes of any size or ploidy go to the very top of the gradient. However, a direct analysis using a fluorescent probe for viability of GPIIb/IIIa-positive cells has shown that the viability in the uppermost fractions, although slightly less than total, is 90%. It is conceivable that the megakaryocytes in these fractions may differ in some fundamental way from other megakaryocytes, although Wright's stained preparations of these cells showed no obvious differences in cytoplasmic granularity or degree of basophilia.

Of particular interest is the evaluation of 2N and 4N cells, cells not readily evident by morphologic features alone. The presence of GPIIb/IIIa on diploid cells is further evidence that this antigen is expressed very early in the megakaryocytic lineage.[13] It is possible that it is expressed as early as the level of the CFU-megakaryocyte since incubation of murine marrow with monoclonal antibodies specific to the human GPIIb/IIIa complex appears to be cytotoxic to these progenitors.[43]

Other investigators have separated human megakaryocytes on Percoll at a density of 1.050 g/mL and have found that virtually all of the megakaryocytes were found at this or lesser density, whereas no megakaryocytes were found in the pellet.[10] On continuous gradients, however, we find that small and low-ploidy megakaryocytes are mainly found at densities greater than 1.050 g/mL. It is not clear whether this discrepancy reflects (1) a greater ability to identify small megakaryocytes with a monoclonal as opposed to polyclonal antibody or (2) differences in separation characteristics between a single Percoll density cut and a continuous gradient. Nevertheless, the possibility exists that a significant proportion of small, low-ploidy megakaryocytes are lost and undetected with a single 1.050-g/mL density cut. This may not be of importance when the sole objective of a study is to purify megakaryocytes to homogeneity for biochemical studies, but may be of considerable importance when examining the response of early megakaryocytes to stimulators of maturation or proliferation.

As has been observed in previous studies, a close relationship exists between megakaryocytic size and ploidy.[46,47] This relationship also applies to 2N and 4N cells. A few of these low-ploidy cells were larger than might be expected. Conceivably, these large cells may represent nonviable high-ploidy cells whose DNA has degraded. A wide range of size distribution is observed at each ploidy class. If the size range expanded as ploidy increased, it is conceivable that a mechanism that regulates cytoplasmic volume exists that is dependent on ploidy. That is, higher ploidy cells may have a greater capacity to vary their cytoplasmic volumes. Examination of the SD of diameter as a function of ploidy as shown in Fig 3 suggests that this is unlikely since there is no apparent trend in size range irrespective of the ploidy class.

In summary, the data presented herein show that the state of megakaryocytic differentiation is inversely related to Percoll density and that a monoclonal antibody to the GPIIb/IIIa complex identifies small and low-ploidy cells of the megakaryocytic lineage unrecognizable by size criteria. The use of a fluorescence method to both identify cells and ascertain their DNA content allows for the precise identification of diploid and tetraploid megakaryocytes. This methodology may be useful for studies of small, low-ploidy megakaryocytes in various pathologic states and the response of these cells to various physiologic and pharmacologic stimuli.

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REFERENCES

1. Metcalf D, MacDonald HR, Odartchenko N, Sordat B: Growth of mouse megakaryocyte colonies in vitro. Proc Natl Acad Sci USA 72:1744, 1975

2. Williams N, McDonald TP, Rabellino EM: Maturation and regulation of megakaryocytes. Blood Cells 5:43, 1979

3. Levine RF, Fedorko ME: Isolation of intact megakaryocytes from guinea pig femoral marrow. Successful harvest made possible with inhibitors of platelet aggregation: Enrichment achieved with a two-step separation technique. J Cell Biol 69:159, 1976

4. Levin J, Levin FC, Metcalf D: The effects of acute thrombocytopenia on megakaryocyte-CFC and granulocyte-macrophage-CFC in mice: Studies of bone marrow and spleen. Blood 56:274, 1980

5. Long MW, Williams N, Ebbe S: Immature megakaryocytes in the mouse: Physical characteristics, cell cycle status, and in vitro responsiveness to thrombopoietic stimulatory factor. Blood 59:569, 1982

6. Vainchenker W, Bonquet J, Guichard J, Breton-Gorius J: Megakaryocyte colony formation from human bone marrow precursors. Blood 54:940, 1979

7. Hoffman R, Mazur E, Bruno E, Floyd V: Assay of an activity in the serum of patients with disorders of thrombopoiesis that stimulates formation of megakaryocytic colonies. N Engl J Med 305:533, 1981

8. Messner HA, Jamal N, Izaguirre C: The growth of large megakaryocyte colonies from human bone marrow. J Cell Physiol 2:45, 1982 (suppl)

9. Kimura H, Burstein SA, Thorning D, Powell JS, Harker LA, Fialkow PJ, Adamson JW: Human megakaryocytic progenitors (CFU-M) assayed in methylcellulose: Physical characteristics and requirements for growth. J Cell Physiol 118:87, 1984

10. Rabellino EM, Nachman RL, Williams N, Winchester RJ, Ross GD: Human megakaryocytes. I. Characterization of membrane and cytoplasmic components of isolated marrow megakaryocytes. J Exp Med 149:1273, 1979

11. Rabellino EM, Levene RB, Leung LLK, Nachman RL: Human megakaryocytes. II. Expression of platelet proteins in early marrow megakaryocytes. J Exp Med 154:88, 1981

12. Mazur EM, Hoffman R, Chasis J, Marchesi S, Bruno E: Immunofluorescent identification of human megakaryocyte colonies using an antiplatelet glycoprotein antiserum. Blood 57:277, 1981

13. Vainchenker W, Deschamps JF, Bastin JM, Guichard J, Titeux M, Breton-Gorius J, McMichael AJ: Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: Immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. Blood 59:514, 1982

14. Damiani G, Zocchi E, Fabbi M, Bargellesi A, Patrone F: A monoclonal antibody to platelet glycoproteins IIb and IIIa complex: Its use in purifying human megakaryocytes from sternal bone marrow aspirates for immunofluorescence studies of Ia-like antigens. Exp Hematol 11:169, 1983

15. Rabellino EM, Levene RB, Nachman RL, Leung LLK: Human megakaryocytes. III. Characterization in myeloproliferative disorders. Blood 63:612, 1984

16. Koike T: Megakaryoblastic leukemia: The characterization and identification of megakaryoblasts. Blood 64:683, 1984

17. Hourdille P, Benabdallah S, Belloc F, Nurden AT: Distribution of glycoprotein IIb-IIIa complexes in the surface membranes of human platelet and megakaryocytes. Br J Haematol 59:171, 1985

18. Levine RF: Isolation and characterization of normal human megakaryocytes. Br J Haematol 45:487, 1980

19. Sitar G: Isolation of normal human megakaryocytes. Br J Haematol 58:465, 1984

20. Jackson CW: Cholinesterase as a possible marker for early cells of the megakaryocytic series. Blood 42:413, 1973

21. Long MW, Williams N: Immature megakaryocytes in the mouse: Morphology and quantitation by acetylcholinesterase staining. Blood 58:1032, 1981

22. Ishibashi T, Burstein SA: Separation of murine megakaryocytes and their progenitors on continuous gradients of Percoll. J Cell Physiol 1985 125:559, 1985

23. Parks DR, Bryan VM, Oi VT, Hertzenberg LA: Antigen-specific identification and cloning of hybridomas with a fluorescence-activated cell sorter (FACS). Proc Natl Acad Sci USA 76:1962, 1979

24. Rotman B, Papermaster BM: Membrane properties on living mammalian cells as studied by enzymatic hydrosis of fluorogenic esters. Proc Natl Acad Sci USA 55:134, 1966

25. Mishell BB, Shiigi SM: In Vitro Immune Responses: Selected Methods in Cellular Immunology. San Francisco, Freeman, 1980, p 19

26. Ault KA, Springer TA: Cross-reaction of a rat antimouse phagocyte-specific monoclonal antibody (anti Mac-1) with human monocytes and natural killer cells. J Immunol 126:359, 1980

27. Jaffe EA, Nachman RI, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins: Identification by morphologic and immunologic criteria. J Clin Invest 52:2745, 1973

28. Ruggeri ZM, de Marco L, Gatti L, Bader R, Montgomery RR: Platelets have more than one binding site for von Willebrand factor. J Clin Invest 272:1, 1983

29. Liu F-T, Bohn JW, Terry EL, Yamamoto H, Molinaro CA, Sherman LA, Klinman NR, Katz DH: Monoclonal anti-dinitrophenyl-specific murine IgE antibody: Preparation, isolation and characterization. J Immunol 124:2728, 1980

30. Clamp JR, Hughes KW, McPherson JC: Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry 8:871, 1971

31. Coller HA, Coller BS: Statistical analysis of repetitive subcloning by the limiting dilution technique with a view toward ensuring hybridoma monoclonality. Hybridoma 2:91, 1983

32. Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry 15:429, 1978

33. Ouchterlony O: Gel diffusion techniques, in Ackroyed JF (ed): Immunological Methods. Philadelphia, Davis, 1964, p 55

34. Kunicki TJ, Pidard D, Rosa J-P, Nurden AT: The formation of Ca^{++}-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. Blood 58:268, 1981

35. Leif RC, Easter HN, Warters RL, Thomas RA, Dunlap LA, Austin MF: Centrifugal cytology. I. A quantitative technique for the preparation of glutaraldehyde-fixed cells for the light and scanning electron microscope. J Histochem Cytochem 19:203, 1971

36. Chatelain C, Burstein SA: Fluorescence cytophotometric analysis of megakaryocytic ploidy in culture: Studies of normal and thrombocytopenic mice. Blood 64:1193, 1984

37. Paulus JM, Penington DG, Kinet-Denoel C, Jackson CW, Odell TT Jr: Determination of megakaryocyte ploidy in human diseases, in Paulus JM (ed): Platelet Kinetics. Amsterdam, Elsevier, 1971, p 330

38. Gogstad GO, Hetland O, Solum O, Prydz H: Monocytes and platelets share the glycoproteins IIb and IIIa that are absent from both cells in Glanzmann's thrombasthenia type I. Biochem J 214:331, 1983

39. Bai Y, Durbin H, Hogg N: Monoclonal antibodies specific for platelet glycoproteins react with human monocytes. Blood 64:139, 1984

40. Clemetson KJ, McGregor JL, McEver RP, Jacques YV, Bainton DF, Domzig W, Baggiolini M: Absence of platelet membrane glycoproteins IIb/IIIa from monocytes. J Exp Med 161:972, 1985

41. Thiagarajan P, Shapiro SS, Levine E, DeMarco L, Yalcin A: A monoclonal antibody to human platelet glycoprotein IIIa detects a related protein in cultured human endothelial cells. J Clin Invest 75:896, 1985

42. Fitzgerald LA, Charo IF, Phillips DR: Human and bovine endothelial cells synthesize membrane proteins similar to human platelet glycoproteins IIb and IIIa. J Biol Chem 260:10893, 1985

43. Berridge MV, Ralph SJ, Tan AS: Cell-lineage antigens of the stem cell-megakaryocyte-platelet lineage are associated with the platelet IIb-IIIa glycoprotein complex. Blood 66:76, 1985

44. Nakeff A, Floeh DP: Separation of megakaryocytes from mouse bond marrow by density gradient centrifugation. Blood 48:133, 1976

45. Rabellino EM: Biology of human megakaryocytes: Recent developments, in Spaet Th (ed): Progress in Hemostasis and Thrombosis, vol 7. Orlando, Fla, Grune & Stratton, 1984, p 151

46. Odell TT Jr, Jackson CW, Friday TJ: Megakaryocytopoiesis in rats with special reference to polyploidy. Blood 35:775, 1970

47. Levine RF, Hazzard KC, Lamberg JD: The significance of megakaryocyte size. Blood 60:1122, 1982

A Soluble Adenosine Triphosphate–Dependent Proteolytic System in Human Peripheral Red Blood Cells

By R.F. Rieder, A. Ibrahim, and J.D. Etlinger

A soluble adenosine triphosphate (ATP)-dependent proteolytic system has been detected in human peripheral blood erythroid cells. Hemolysates prepared from reticulocyte-rich blood of subjects with autoimmune hemolytic anemia, treated pernicious anemia, and iron deficiency anemia or from pools of red blood cells enriched for reticulocytes by density gradient centrifugation were tested against a radioactive casein standard. Up to 57% of the casein was rendered trichloroacetic acid (TCA) soluble after incubation with such hemolysates for 60 minutes in the presence of 1.0 mmol/L ATP. In the absence of ATP or in hemolysates prepared from reticulocyte-poor blood as little as 6% to 10% of the casein was hydrolyzed. The proteolytic activity was found in the 100,000-g supernatant of active hemolysates and was blocked by hemin, *N*-ethylmaleimide, and sodium vanadate and thus resembles a previously described activity in rabbit reticulocytes. In the presence of ATP, similar lysates prepared from rabbit reticulocytes preferentially hydrolyzed the abnormal human hemoglobins Leiden and Gun Hill compared with hemoglobin A. These results suggest that there is an active ATP-dependent proteolytic system in young human erythroid cells that can degrade certain abnormal globin chains; the enzymatic activity is lost in the transition from reticulocyte to erythrocyte.

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PROTEIN BREAKDOWN is a constant process within bacteria and animal cells.[1-4] The rates of proteolysis as well as the rates of synthesis determine the concentrations of various proteins, those with abnormal structure being degraded especially rapidly.[3,4] A soluble adenosine triphosphate (ATP)-dependent proteolytic system has been described in cell-free preparations from rabbit reticulocytes.[5] This proteolytic activity appears to be especially active against denatured or abnormal proteins. Upon maturation of the reticulocyte, the ATP-dependent proteolysis is gradually lost from the rabbit cells.[6-8] Examination of more primitive erythroid cells, the Friend mouse erythroleukemia (MEL) and the human K562 erythroleukemia cell lines, has also revealed the presence of ATP-dependent proteolytic enzyme systems.[9] In the two erythroleukemia cell lines, we found the ATP-dependent activity to be associated with a particulate fraction sedimentable at 100,000 g,[9] although soluble activity also has been reported recently in MEL cells.[10]

In human erythroid cells there is postsynthetic degradation of certain unstable abnormal hemoglobins as well as unpaired excess globin chains produced in the thalassemias.[11-15] The proteolytic mechanism responsible for this phenomenon has not been defined. We have examined human reticulocyte-rich peripheral blood for the presence of an ATP-dependent proteolytic activity. An active system has been demonstrated in cell-free erythrocyte extracts; as in the analogous system in rabbit reticulocytes, the activity in human peripheral red blood cells is soluble and markedly declines with erythrocyte maturation.

MATERIALS AND METHODS

Rabbit reticulocytes were isolated as previously described from phenylhydrazine-treated animals.[5] Human peripheral venous blood was anticoagulated with EDTA. The red blood cells were washed three times with 0.1 mmol/L NaCl (Fisher Scientific, Springfield, NJ), 15 mmol/L KCl (Fisher), 25 mmol/L Tris, pH 7.8, and lysed with 1.6 vol of hypotonic buffer (10 mmol/L Tris, pH 7.8, containing 0.5 mmol/L dithiothreitol). Leupeptin (Sigma Chemical Co, St Louis), 10 μg/mL, was added to stabilize ATP-dependent proteolysis. The cells were then homogenized using the A and B pestles of a Dounce homogenizer (Thomas Scientific, Swedesboro, NJ). The hemolysate was centrifuged at 15,600 g for 15 minutes, followed by centrifugation of the supernatant at 100,000 g for 60 minutes. Twenty-five microliters of the 100,000-g supernatant were assayed for proteolytic activity in a 50-μm reaction mixture in the presence or absence of 1 mmol/L ATP and an ATP-generating system consisting of 50 μg/mL creatine phosphokinase and 12 mmol/L creatine phosphate as previously described.[7] An ATP trap consisting of 10 mmol/L glucose and 0.5 units of hexokinase was added to reaction mixtures lacking added ATP. Hemoglobin Gun Hill[16] and hemoglobin Leiden[12] were each isolated by diethyl aminoethyl (DEAE)-cellulose chromatography from the blood of subjects heterozygous for these unstable hemoglobins. In each case, hemoglobin A was also prepared from the same blood. The hemoglobins and α-casein were used as proteolytic substrates at a concentration of 5 μmol/L after being rendered radioactive by reductive methylation using ^{14}C-formaldehyde (0.25 mCi in 0.14 mg, New England Nuclear, Boston) followed by treatment with sodium borohydride as described previously.[17] The generation of trichloroacetic acid (TCA)-soluble radioactivity was measured, and the degradation of labeled protein was expressed as the percentage of the acid-soluble counts divided by the added acid-precipitable counts × 100.

RESULTS

A 42-year-old woman with an exacerbation of Coomb's positive autoimmune hemolysis (AIHA) had a decrease in the packed red blood cell volume to 19% and an increase in reticulocytes to 21%. Therapy with prednisone was instituted, and over the course of 2 months hemolysis declined, the hemoglobin level rose, and the reticulocyte count fell (Fig 1). During the early portion of the course of therapy when the reticulocyte count varied from 22% to 13%, the 100,000-g supernatant of the red cell lysate hydrolyzed 26% to 37% of a standard preparation of radioactive casein in the presence of 1 mmol/L ATP during 60 minutes of incubation at 37 °C (Fig 1). In the absence of ATP, 6.9% to 9.5% of the standard was

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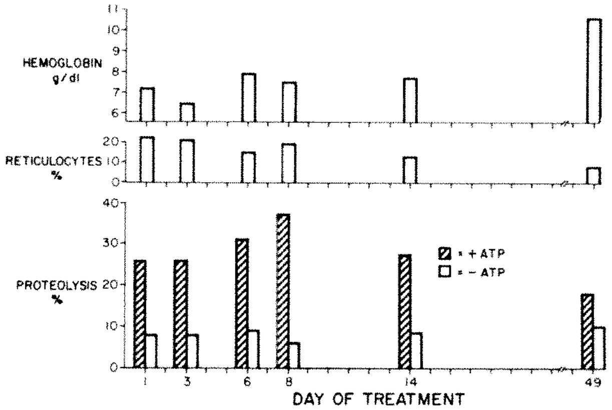

Fig 1. Proteolysis by erythrocyte lysates during the course of recovery from an episode of AIHA. The hemoglobin and reticulocyte levels as well as the proportion of casein digested in the presence and absence of 1.0 mmol/L ATP are shown for a 49-day period.

rendered TCA soluble. In some preparations stimulation of proteolysis by ATP was as high as sixfold. Similar lysates prepared from two normal control subjects hydrolyzed, respectively, 10.7% and 9.7% of the casein in the presence of ATP and 6.9% and 7.1% without ATP (Table 1). Recovery of the patient from the hemolytic process was associated with a decline in ATP-dependent proteolysis. When the packed cell volume had risen to 34% and the reticulocyte count had fallen to 8.4% per cent, the 100,000-g supernatant of the red cell lysate hydrolyzed 18% of the casein standard with ATP and 10% without AtP (Fig 1). When the reticulocyte count reached 4.6% proteolysis was reduced to 12.0% with ATP and 6.1% without ATP (data not shown). A precise correlation between the number of reticulocytes and the degree of proteolysis was not observed in these studies, nor would it be expected using a nonpurified enzyme preparation derived from heterogeneous, nonsynchronized cell populations.

The time course of in vitro proteolysis was examined when the subject's blood contained 19.1% reticulocytes. Proteolysis of the casein substrate was assessed at several points during 120 minutes of incubation. In the presence of ATP, hydrolysis of the substrate was most rapid for the first 30 minutes (Fig 2). By 60 minutes, 30% of the substrate had been hydrolyzed with ATP and 11.1% without ATP. Hydrolytic activity declined for the next 60 minutes; by 120 minutes, 34.8% of the substrate had been hydrolyzed in the presence of ATP and 14.1% in the absence of ATP (Fig 2). Exhaustion of some component of the reaction mixture could account for this decline in the rate.

Table 1. ATP-Dependent Proteolysis in Some Subjects With Reticulocytosis

Subject	Reticulocytes (%)	Proteolysis (%)	
		+ATP	−ATP
Treated iron deficiency anemia	10.0	15.9	9.8
Treated pernicious anemia	28.5	28.6	11.5
Normal	<1.0	10.7	6.9
Normal	<1.0	9.7	7.1

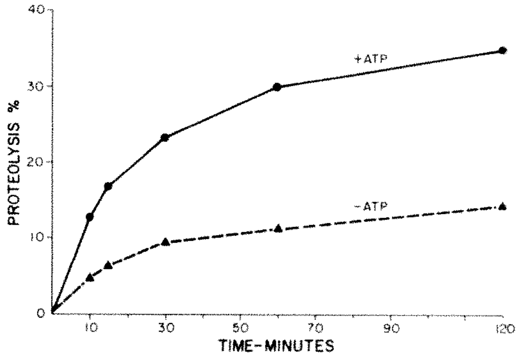

Fig 2. Time course of proteolysis in vitro by a peripheral red blood cell lysate. The proportion of casein digested for a period of 120 minutes in the presence (●—●) and absence (▲---▲) of 1.0 mmol/L ATP is shown for a lysate prepared from the blood of the patient with AIHA when the reticulocyte count was 19.1%.

Because the activity of the ATP-dependent proteolytic system in the peripheral blood cells declined with the decrease in reticulocyte count during the course of treatment and because normal subjects with low reticulocyte counts showed little ATP-dependent activity, an attempt was made to fractionate circulating red blood cells by age to examine changes in ATP-dependent proteolytic activity with erythrocyte maturation.

During the process of aging, peripheral red blood cells are known to become increasingly dense. Blood from the patient with AIHA was obtained when the reticulocyte count was 24.7%. The red blood cells were adjusted to a hematocrit value of 50% in plasma and mixed with 20 vol of a solution containing colloidal silica (Percoll, Pharmacia Fine Chemicals, Piscataway, NJ), 60% meglumine diatrizoate (Hypaque, Winthrop Laboratories, New York), H_2O, and 0.9% NaCl in the proportion of 3.5:1.7:4.1:0.7.[18] The mixture was centrifuged at 32,000 g for 20 minutes at room temperature. The resultant multiple erythrocyte bands were collected in three pools, top, middle, and bottom, that contained 67%, 5.5%, and 0% reticulocytes, respectively. Lysates prepared from the three erythrocyte fractions were assayed for proteolytic activity against the radioactive casein standard after 60 minutes of incubation at 37 °C (Fig 3). The least dense fraction of red cells, containing the bulk of the reticulocytes, demonstrated marked (55.7%) ATP-dependent proteolytic activity (Fig 3). Stimulation of proteolysis by ATP was 4.4-fold. Much less ATP-dependent proteolysis (13.3%) was observed in the bottom, dense erythrocyte fraction containing 0% reticulocytes (Fig 3). When normal red blood cells were subjected to density gradient centrifugation, the top fraction exhibited 15.6% proteolysis in the presence and 13.0% in the absence of ATP.

Several enzyme inhibitors were studied for their effect on the ATP-dependent proteolytic system (Table 2). Hemin, the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM, a sulfhydryl reagent), and the ATPase inhibitor sodium vanadate[19] all markedly depressed ATP-dependent proteolysis by a hemolysate from the subject with AIHA. Except for the observed inhibitory

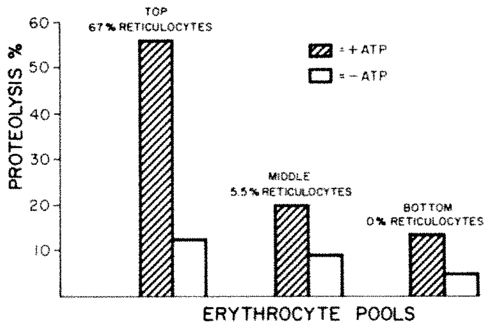

Fig 3. Proteolysis by erythrocytes of different densities. Red blood cells were collected from the subject with AIHA at a time when the reticulocyte count was 24.7%. The erythrocytes were centrifuged at 32,000 g for 20 minutes in a mixture of colloidal silica, 60% meglumine diatrizoate, water, and 0.9% NaCl (see text for details). Three pools of erythrocytes were collected from the top, middle, and bottom portions of the resultant gradient and contained 67%, 5.5%, and 0% reticulocytes, respectively. The digestion of the casein standard in the presence and absence of 1.0 mmol/L ATP is shown for the lysates prepared from the three pools of red blood cells.

effect of PMSF, these results are similar to those previously described in studies of the rabbit reticulocyte system[5,8] and the particle-associated ATP-dependent proteolytic activity of mouse (MEL) and human (K562) erythroleukemia cells.[9]

Other subjects with elevated reticulocyte counts were studied for the presence of an ATP-dependent proteolytic activity in the peripheral blood (Table 1). A patient with iron deficiency anemia undergoing treatment with iron who had 10% reticulocytes demonstrated digestion of 15.9% of the substrate in the presence of ATP and 9.8% in the absence of ATP. A subject with pernicious anemia responding to vitamin B_{12} who had 28.5% reticulocytes showed 28.6% proteolysis in the presence of ATP and 11.5% in the absence of ATP (Table 1).

It has been suggested that proteolytic enzymes within red blood cells may serve in the removal of denatured abnormal hemoglobins that are harmful to the cell. The ability of the ATP-dependent proteolytic enzyme to hydrolyze unstable hemoglobin Gun Hill[16] and hemoglobin Leiden[12] was examined. The mutant hemoglobins as well as hemoglobin A were separated by column chromatography on DEAE-cellulose[12] from the blood of two heterozygous subjects and labeled by reductive methylation.[17] The abnormal radioactive hemoglobins were compared with radioactive hemoglobin A for their susceptibility to hydrolysis by a reticulocyte lysate prepared from rabbit blood (Fig 4). Proteolysis of both hemoglobin Gun Hill and hemoglobin Leiden was increased in the presence of ATP. Moreover, both of these abnormal unstable hemoglobins were more sensitive to proteolysis than hemoglobin A (Fig 4). Hydrolysis of the abnormal hemoglobins was suppressed by the addition of 0.5 mmol/L hemin to the incubation mixture (Fig 4).

Table 2. Effect of Various Enzyme Inhibitors on ATP-Dependent Proteolysis by a Hemolysate From a Subject With AIHA

Inhibitor (mmol/L)	Proteolysis (%)	
	+ATP	−ATP
None	19.7	7.3
Hemin 0.05	7.9	5.4
PMSF 1.0	10.0	5.9
NEM 5.0	3.0	2.1
Na vanadate 5.0	6.1	5.9

PMSF, phenylmethylsulfonyl fluoride; NEM, *N*-ethylmaleimide.

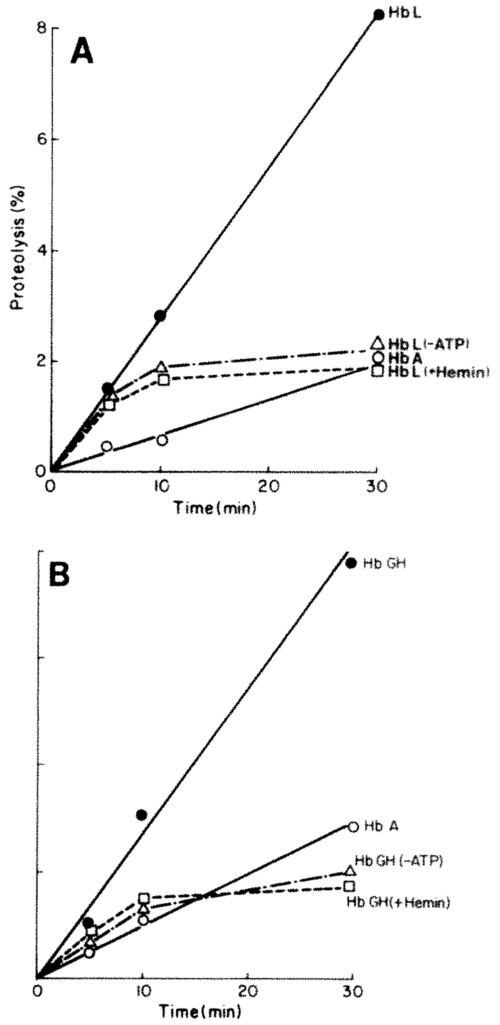

Fig 4. Hydrolysis of hemoglobins A, Leiden, and Gun Hill by rabbit reticulocyte lysates. In the presence of ATP there was enhanced proteolysis of the unstable hemoglobin Leiden (A) and hemoglobin Gun Hill (B) compared with normal hemoglobin A. The decreased amount of proteolysis of the unstable hemoglobins in the absence of ATP as well as the inhibition of ATP-enhanced proteolysis by hemin is shown.

DISCUSSION

These studies demonstrate an active proteolytic enzyme system in cell-free preparations of human peripheral red blood cells. The enzyme is active in vitro at neutral pH and is stimulated by the presence of 1.0 mmol/L ATP. Blood from subjects with elevated reticulocyte counts exhibit high levels of the ATP-dependent proteolytic activity that declines with a decrease in the degree of reticulocytosis. Little activity is demonstrable in normal reticulocyte-poor blood. Density gradient separation of peripheral erythrocytes also clearly indicated that the enzyme system is most active in the young reticulocyte-rich cell fractions and essentially absent in dense, older red blood cells. The enzyme is inhibited by hemin, PMSF, and the sulhydryl reagent, NEM. Hydrolysis of ATP is probably required for enzyme activity because proteolysis is markedly reduced by the ATPase inhibitor sodium vanadate.

Previous studies of hemoglobin synthesis in vitro have demonstrated that the intact human erythroid cell is able to degrade abnormal unstable hemoglobins as well as the unpaired globin chains that occur in the thalassemia syndromes.[11-15] A proteolytic mechanism has been suspected of being responsible for this activity. In red blood cells from patients with β-thalassemia, in vitro turnover of excess α-globin chains has been shown to decline when the cells are deprived of energy and the ATP levels fall.[20] The ATP-dependent proteolytic system derived from rabbit erythrocytes has been shown previously to be capable of distinguishing between artifically induced abnormal and normal hemoglobin.[5] Although we have not yet studied this capacity in the human enzyme system, the present experiments with rabbit reticulocyte lysates demonstrated that the unstable human hemoglobins Gun Hill and Leiden were preferentially hydrolyzed compared with hemoglobin A.

The enzyme system described here in human reticulocytes seems to be analogous to the activity found in rabbit reticulocytes. The human system is sensitive to the same series of enzyme inhibitors and is also most active in reticulocytes, declining in older red blood cells. The activity demonstrated in the human reticulocytes also is limited to the soluble portion of the cell lysate. In this characteristic it differs from a previously described ATP-dependent proteolytic system found in certain more primitive erythroid cells. In both MEL and K562 cells an ATP-dependent proteolytic system with characteristics otherwise quite similar to the soluble system in reticulocytes has been found associated with a subcellular fraction sedimentable at 100,000 g.[9] Recently Waxman and associates found soluble ATP-dependent activity in MEL cells.[10] The relationship of the soluble to the insoluble activity is not understood at present. It is conceivable that the activity in young nucleated erythroid precursors is associated initially with a membrane or particle fraction and during cell differentiation becomes solubilized. Finally, upon further cell maturation the activity might be lost entirely. Further studies are planned to test this hypothesis. A similar change in physical state has been previously suggested for the erythrocyte NADH–cytochrome b_5 reductase (methemoglobin reductase).[21]

There are several earlier reports of other proteolytic activity detected in extracts of human erythroid cells. Lewis and Harris[22] described a group of peptidases in lysates of peripheral erythrocytes that were separable by electrophoresis. These enzymes exhibited differences in substrate specificity; genetic variants were also observed. Hanash and Rucknagel[23] reported that lysates prepared from thalassemia marrow and peripheral blood normoblasts could, upon addition to radioactive lysates of β-thalassemia erythrocytes, reduce the level of excess α-globin radioactivity. Vettore et al[24] also reported that after incubation of peripheral blood from both thalassemic and nonthalassemic subjects with tritiated leucine a further 24-hour incubation of crude hemolysates resulted in the loss of approximately 30% of the α-globin radioactivity. This loss of radioactivity was blocked by an inhibitor of chymotrypsinlike proteases, tosyl-amido-phenylethyl-chloromethyl-ketone (TPCK). In that report the extensive loss of the α-globin radioactivity even from hemolysates of normal blood is surprising since presumably the radioactive α-globin was incorporated into completed hemoglobin tetramers that are relatively stable.

A proteolytic activity associated with the erythrocyte membrane was described by Ballas and Burka.[25] Melloni and associates have extensively investigated several distinct proteolytic activities found in human red blood cells.[26-28] None of the enzymes studied required energy, and there was only a relatively modest decline in activity during the period of the reticulocyte-erythrocyte transition.[27] A calcium-dependent neutral protease was detected in erythrocyte cytosol that was inhibited by hemin, but unlike the ATP-dependent activity described in the present paper, it was also markedly inhibited by leupeptin.[28] Membrane-bound acid proteases optimally active at approximately pH 2.5 were also found.[26,29] In the present experiments some proteolytic activity was always detected even in the absence of ATP. However, especially in lysates prepared from cell samples containing high proportions of reticulocytes, the activity present without ATP was only a fraction of the ATP-dependent activity (Fig 2).

In rabbit reticulocytes, ubiquitin, a heat-stable polypeptide of 76 amino acids, becomes covalently attached to proteins through the action of activating and conjugating enzymes.[30] The activation of ubiquitin requires ATP and Hershko and associates[30] have proposed that ubiquitin conjugation to substrate proteins acts as a tag for protease recognition. In contrast, Speiser and Etlinger[31] have provided evidence that ubiquitin acts to release the protease from an endogenous inhibitor. The role of ubiquitin in human reticulocytes has not been studied.

Several other ATP-dependent proteolytic systems have been described in bacteria,[1,32] eukaryotic cytosol preparations,[33] and mitochondria.[34] The function of these enzymes in the economy of the various cells is not clearly understood, but it has been suggested that they could play a role in such functions as regulation of the activity of other short-lived enzymes in growth and development in addition to providing a pathway for removal of polypeptides with abnormal structures. In rabbit reticulocytes the ATP-dependent system is also thought to be responsible for the programmed degradation of proteins that is associated with the loss of mitochondria, ribosomes, and cytosolic enzymes during cell development.[8]

The role of the ATP-dependent proteolytic system in human red blood cells requires further study. Unstable hemoglobins and free unpaired globin chains tend to precipitate within erythroid cells to form Heinz bodies. These cellular inclusion bodies may attach to the band 3 component of the cell membrane causing damage and stimulating erythrophagocytosis by macrophages.[35-37] An enzyme system capable of removing precipitated hemoglobin might have a salutory effect on red cell survival. Thus the ATP-dependent system could modulate disease severity in both the thalassemias and the unstable hemoglobinopathies.

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REFERENCES

1. Mount DW: The genetics of protein degradation in bacteria. Annu Rev Genet 14:279, 1980
2. Hershko A, Chiechanover A: Mechanisms of intracellular protein breakdown. Annu Rev Biochem 51:335, 1982
3. Goldberg AL, Dice JF: Intracellular protein degradation in mammalian and bacterial cells. Annu Rev Biochem 43:835, 1974
4. Goldberg AL, St. John AC: Intracellular protein degradation in mammalian and bacterial cells: Part 2. Annu Rev Biochem 45:747, 1976
5. Etlinger JD, Goldberg AL: A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. Proc Natl Acad Sci USA 74:54, 1977
6. Muller M, Dubiel W, Rathmann J, Rapaport S: Determination and characteristics of energy-dependent proteolysis in rabbit reticulocytes. Eur J Biochem 109:405, 1980
7. Speiser S, Etlinger JD: Loss of ATP-dependent proteolysis with maturation of reticulocytes and erythrocytes. J Biol Chem 257:14122, 1982
8. Boches FS, Goldberg AL: Role for the adenosine triphosphate-dependent proteolytic pathway in reticulocyte maturation. Science 215:978, 1982
9. Rieder RF, Ibrahim A, Etlinger JD: A particle-associated ATP-dependent proteolytic activity in erythroleukemia cells. J Biol Chem 260:2015, 1985
10. Waxman L, Fagan JM, Tanaka K, Goldberg AL: A soluble ATP-dependent system for protein degradation from murine erythroleukemia cells. J Biol Chem 260:11994, 1985
11. Rieder RF: Human hemoglobin stability and instability: Molecular mechanisms and some clinical correlations. Semin Hematol 11:423, 1974
12. Rieder RF, James GW III: Imbalance in α and β globin synthesis associated with a hemoglobinopathy. J Clin Invest 54:948, 1974
13. Chalevelakis G, Clegg JB, Weatherall DJ: Imbalanced globin chain synthesis in heterozygous β-thalassemia bone marrow. Proc Natl Acad Sci USA 72:3853, 1975
14. Wood WG, Stamatoyannopoulos G: Globin synthesis in fractionated normoblasts of β-thalassemia heterozygotes. J Clin Invest 55:567, 1975
15. Sancar GB, Cedeno MM, Rieder RF: Rapid destruction of newly synthesized excess β-globin chains in HbH disease. Blood 57:967, 1981
16. Rieder RF, Bradley TB Jr: Hemoglobin Gun Hill: An unstable protein associated with chronic hemolysis. Blood 32:355, 1968
17. Rice RH, Means GE: Radioactive labelling of proteins in vitro. J Biol Chem 246:831, 1971
18. Fabry ME, Kaul DK, Raventos C, Baez S, Rieder RF, Nagel RL: Some aspects of the pathophysiology of homozygous HbCC erythrocytes. J Clin Invest 67:1284, 1981
19. Cantley LC Jr, Josephson L, Warner R, Yanagisawa, Lechene C, Gridotti G: Vanadate is a potent (Na,K)-ATPase inhibitor found in ATP derived from muscle. J Biol Chem 252:7421, 1977
20. Shaeffer JR: Turnover of excess hemoglobin α chains in β-thalassemic cells is ATP-dependent. J Biol Chem 258:13172, 1983
21. Choury D, Leroux A, Kaplan JD: Membrane-bound cytochrome b_5 reductase (methemoglobin reductase) in human erythrocytes. J Clin Invest 67:149, 1981
22. Lewis WHP, Harris H: Human red cell peptidases. Nature 215:351, 1967
23. Hanash SM, Rucknagel DL: Proteolytic activity in erythrocyte precursors. Proc Natl Acad Sci USA 75:3427, 1973
24. Vettore L, DeMatteis MC, DiLorio EE, Winterhalter KH: Erythrocyte proteases: Preferential degradation of alpha hemoglobin chains. Acta Haematol 70:35, 1983
25. Ballas SK, Burka ER: Catabolism of hemoglobin by human erythrocyte membranes. J Lab Clin Med 92:387, 1978
26. Melloni E, Salamino F. Sparatore B, Michetti M, Pontremoli S: Cooperation between soluble and membrane-bound proteinases in the degradation of β-hemoglobin chains in intact human erythrocytes. Arch Biochem Biophys 216:495, 1982
27. Melloni E, Salamino F, Sparatore B, Michetti M, Niorelli A, Benatti V, DeFlora A, Pontremoli S: Decay of proteinase and peptidase activities of human and rabbit erythrocytes during cellular aging. Biochim Biophys Acta 675:110, 1981
28. Melloni E, Sparatore B, Salamino F, Michetti M, Pontremoli S: Cytosolic calcium dependent proteinase of human erythrocytes: Formation of an enzyme-natural inhibitor complex induced by Ca^{2+} ions. Biochem Biophys Res Commun 106:731, 1982
29. Pontremoli S, Melloni E, Sparatore B, Michetti M, Horecker BL: A dual role for the Ca^{2+}-requiring proteinase in the degradation of hemoglobin by erythrocyte membrane proteinases. Proc Natl Acad Sci USA 81:6714, 1984
30. Hershko A, Chiechanover A, Heller H, Haas AL, Rose IA: Proposed role of ATP in protein breakdown; conjugation of proteins with multiple chains of the polypeptide of ATP-dependent proteolysis. Proc Natl Acad Sci USA 77:1783, 1980
31. Speiser S, Etlinger JD: ATP stimulates proteolysis in reticulocyte extracts by repressing an endogenous inhibitor. Proc Natl Acad Sci USA 80:3577, 1983
32. Chung CH, Waxman L, Goldberg AL: Studies of the protein encoded by the Lon mutation, Cap R9 in *Escherichia coli*. J Biol Chem 258:215, 1983
33. DeMartino GN, Goldberg AL: Identification and partial purification of an ATP-stimulated alkaline protease in rat liver. J Biol Chem 254:3712, 1979
34. Desautels M, Goldberg AL: Liver mitochondria contain an ATP-dependent, vanadate-sensitive pathway for the degradation of proteins. Proc Natl Acad Sci USA 79:1869, 1982
35. Flynn TP, Allen DW, Johnson GJ, White JG: Oxidant damage of the lipids and proteins of the erythrocyte membranes in unstable hemoglobin disease. J Clin Invest 71:1215, 1983
36. Low PS, Waugh SM, Zinke K, Drenckhahn D: The role of hemoglobin denaturation and band 3 clustering in red blood cell aging. Science 227:531, 1985
37. Waugh SM, Low PS: Hemichrome binding to band 3: Nucleation of Heinz bodies on the erythrocyte membrane. Biochemistry 24:34, 1985

High-Dose Melphalan With Autologous Bone Marrow Transplantation for Multiple Myeloma

By Bart Barlogie, Roy Hall, Axel Zander, Karel Dicke, and Raymond Alexanian

A large dose of melphalan was given to 23 patients with advanced multiple myeloma that was refractory to multiple prior treatments. Sixteen patients received a dose of 80 to 100 mg/m^2, and seven were given 140 mg/m^2 followed by autologous bone marrow infusion. Tumor mass was reduced by more than 75% in 14 patients, including four who died of bone marrow aplasia. Serious infections were prevented in six of seven patients who received autologous bone marrow. The marked cytoreduction in patients with previously refractory disease indicated that apparent drug resistance could be overcome by dose escalation. However, short remission times in most responding patients were consistent with rapid regrowth of primordial tumor cells with high proliferative activity. Although high-dose melphalan was of limited benefit to patients with refractory myeloma, further studies are necessary to clarify its role during earlier phases of disease.

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UNTIL RECENTLY, few treatment programs have been effective in patients with multiple myeloma resistant to alkylating agent–prednisone combinations. Using frequent courses of glucocorticoids with vincristine and doxorubicin by continuous infusion (VAD), about one half of our patients achieved a marked tumor reduction.[1] These results indicated that both dose and schedule modification of standard drugs would enhance tumor cell kill, a finding that was supported by the marked cytoreduction achieved in most patients with very high doses of melphalan.[2] To improve the results from salvage therapies, we evaluated high doses of melphalan in 23 patients with advanced multiple myeloma that was resistant to both standard melphalan and the VAD regimen. Sixty percent of our patients showed rapid and marked tumor reductions. Severe and prolonged bone marrow depression caused the death of about one third of our patients, a complication usually prevented by autologous bone marrow infusion.

MATERIALS AND METHODS

A large dose of melphalan was given intravenously to 23 patients with advanced refractory myeloma (Table 1). Lower doses of 80 mg/m^2 (one 74-year-old patient) and 100 mg/m^2 (15 patients) were given as a single 30-minute infusion. When the limited efficacy and severe toxicity of this program were appreciated, a higher dose of 140 mg/m^2 was delivered in two daily fractions followed by the infusion of 12 to 16 × 10^9 autologous marrow cells one day later (seven patients). Autologous bone marrow had been stored during VAD-induced second remissions in six patients and following ineffective VAD treatment in one patient with only extramedullary disease. Bone marrow was collected under general anesthesia, frozen at 1 °C/min and kept at −196 °C for 4 to 27 months. CFU-GM of the stored marrows ranged from eight to 92 colonies per 10^5 nucleated cells (median, 21). Plasma cell counts ranged from 2% to 7% (median, 3%). All patients were admitted to the hospital, and seven were treated in a protected environment unit under strict gnotobiotic conditions. Antibiotic prophylaxis was performed with trimethoprim-sulfamethoxazole and ketoconazole. Patients were advised of procedures and attendant risks in accordance with institutional guidelines and gave informed consent.

The median age of patients treated with melphalan alone was 44 years, whereas the age of those who also received autologous marrow was 63 years. All patients were resistant to multiple prior chemotherapy regimens, and all but two were refractory to salvage VAD therapy.[1] Ten patients had never responded to any prior treatment, and 13 were relapsing to usually two prior regimens. All but three had a high or intermediate tumor mass by standard staging criteria. Flow cytometric (FCM) analyses of bone marrow samples for DNA and RNA content of plasma cells were conducted as described previously.[3] Unfavorable cytometric features included the presence of hypodiploid and biclonal DNA stemlines and/or a RNA content of <4 times that of normal lymphocytes.[4] With any of these features, only 20% of previously untreated patients and less than 10% of patients with resistant myeloma had responded to VAD.[1,5] Ten of our patients had such unfavorable FCM features, previously recognized by multivariate regression analysis to represent the single most important variable both for initial remission[6] and to response to salvage treatment.[1]

Serial studies were conducted to monitor antitumor effect and toxicity. A significant response was defined by a reduction in tumor mass (serum myeloma protein) exceeding 75% with the disappearance of Bence Jones protein excretion for at least 2 months.[7] Five patients died within 6 weeks (early deaths), four of them with a marked antitumor effect comparable to that observed in responding patients. Relapse-free and overall survival times were calculated by life table analyses from the onset of treatment.

RESULTS

Ten of 23 patients who received high-dose melphalan responded for at least 2 months; four other patients responded, but were rated as failures because they died between 4 and 6 weeks from infection due to neutropenia (Table 2). Tumor-halving times calculated from changes in serum myeloma protein levels were very short in all responding patients (median, 0.3 months), with a tumor mass reduction in four patients to levels lower than those observed after any prior therapy. All patients considered responsive showed clearing of bone marrow plasma cells (<7%). Patients with or without response had similar features in terms of tumor mass and immunoglobulin type. Likewise, the incidence of response was not affected by treatment conditions such as the protective environment or autologous bone marrow transplantation. Although only one of 13 patients with unfavorable FCM features had responded to VAD previously, seven of ten patients with such abnormalities achieved a marked cytoreduction with high-dose melphalan ($P < .01$, chi-square test) (Table 2). Similarly, five of ten previously unresponsive

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Table 1. High-Dose Melphalan in Refractory Myeloma

	Autologous Bone Marrow Support		
Characteristic	No	Yes	Total
N	16	7	23
Age (yr)			
Range	24-74	35-66	24-74
Median	44	63	54
Treated in protected environment	4	3	7
No. prior treatment regimens			
Range	1-7	3-6	1-7
Median	3	3	3
Prior VAD salvage treatment	14	7	21
No prior response	8	2	10
Unfavorable FCM features	9	2	11
Intermediate and high tumor mass	14	6	20
Treatment outcome			
Response	6	4	10
Early death*	4	1	5
Failure†	6	2	8

Abbreviations: FCM, flow cytometric.

*At ≤6 weeks from high dose melphalan.

†Survived >6 weeks from high-dose melphalan and achieved <75% tumor mass reduction.

patients experienced a marked antitumor effect with high-dose melphalan in contrast with a 20% response rate for similar patients receiving VAD. The median survival of all patients was 4 months (Fig 1), and the median relapse-free survival of our ten responders was 4 months (range, 2.5 to 18+ months). Four patients are still alive (3, 6, 13, 20 months), including two patients who received bone marrow support.

The major complication was marked and prolonged agranulocytosis and severe thrombocytopenia to less than 10,000/μL for a median of 4 weeks that required a median of 40 units of transfused platelets per patient. With bone marrow transplantation and despite a higher melphalan dose, minimal leukocyte (but not granulocyte or platelet) recovery to at least 200/μL proceeded significantly faster and more uniformly than in the other 16 patients not receiving bone marrow support ($P < .05$, Wilcoxon test) (Fig 2). Further hematologic recovery with granulocytes greater than 1,500/μL and platelets greater than 150,000/μL occurred in 11 and seven of 16 patients without marrow support and in six and three of seven patients who received autologous marrow.

As a result of severe and persistent neutropenia in all 16 patients without marrow support, four patients with a marked antitumor effect and one patient with resistant myeloma died of disseminated fungal infection (Table 3). An additional patient, still in complete remission of his myeloma, died of *Escherichia coli* sepsis resulting from secondary marrow aplasia after previous complete hematologic recovery. The high incidence of suspected or proven fungal infection led to amphotericin B treatment in 11 of 16 patients. In contrast, the only death among the seven patients

Table 2. Response to High-Dose Melphalan According to Pretreatment Features

Prior Response	N	Nucelic Acid Features		Total	Percentage
		Favorable	Unfavorable		
Yes	13	5/8	1(4)/5	6(9)/13	46(69)
No	10	1(2)/4	3/6	4(5)/10	40(50)
Total	23	6(7)/12	4(7)/11	10(14)/23	
Percentage	100	50(58)	36(64)		43(61)

Parentheses include patients who died with >75% tumor cytoreduction.

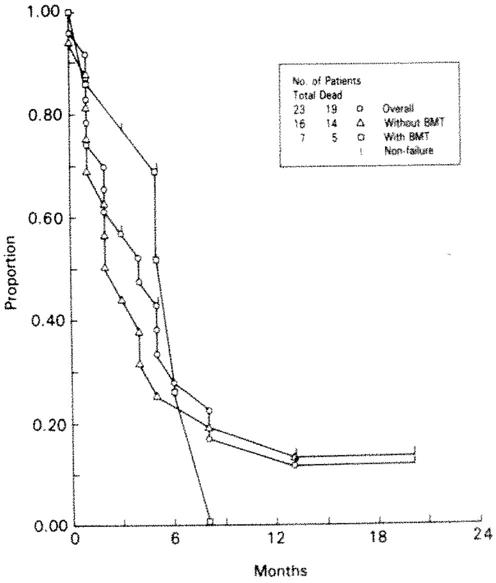

Fig 1. **Kaplan-Meier survival plots in patients with refractory myeloma receiving high-dose melphalan with (□) or without (△) autologous bone marrow. The melphalan dose was higher in the marrow support group (140 mg/m²) compared with patients not receiving autografts. There was only one of seven drug-related deaths in the marrow transplant group compared with six of 16 among the remaining patients (see also Table 3). Four patients remain alive at 3, 6, 13, and 20 months.**

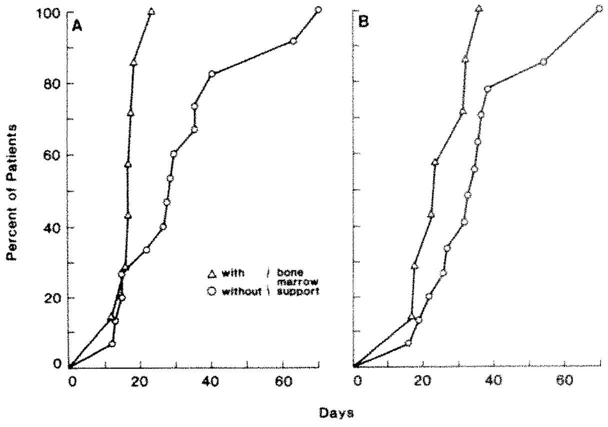

Fig 2. **Peripheral blood leukocyte recovery with and without bone marrow transplantation following high-dose melphalan treatment for refractory myeloma (for further details, see text and Fig 1). Even though the time course to complete hematologic recovery was similar in both groups, patients receiving marrow support demonstrated a faster and more uniform leukocyte (but not granulocyte or platelet) recovery to 200 and 500/μL, preventing death from neutropenic sepsis in all but one transplant recipient.**

Table 3. High-Dose Melphalan in Refractory Myeloma

Marrow Support	Antitumor Effect	N	Alive	Causes of Death: Melphalan-Related	Causes of Death: Progressive Disease
No	Yes	10	1	5	4
	No	6	1	1	4
Total		16	2	6	8
Yes	Yes	4	1	0	3
	No	3	1	1	1
Total		7	2	1	4

receiving marrow infusion occurred in a patient with agranulocytosis persisting beyond day 35 after treatment who died of *Pseudomonas* sepsis. This patient had received prior high-dose cyclophosphamide on two occasions prior to marrow storage.

Thus, although their median age was 20 years less, six of seven treatment-related deaths occurred among the patients who did not receive bone marrow support (Table 3). Other toxicities included reversible hemorrhagic episodes in eight, diarrhea in 13, and pancreatitis in two patients.

DISCUSSION

The median survival of patients with multiple myeloma is approximately 30 months, with both the pretreatment tumor load and the sensitivity to chemotherapy representing the major prognostic factors.[8] Until recently, the likelihood of controlling myeloma resistant to melphalan-prednisone has been less than 20%.[9] Although about 50% of the patients responded to the VAD regimen,[1] those who remain unresponsive or relapse to this program present a difficult problem. The observation by McElwain and Powles that a high dose of melphalan induced a marked degree of remission in all nine patients including five who were resistant to standard melphalan seemed promising.[2] We evaluated this program in a group of patients who were older, had received extensive prior chemotherapy including doxorubicin, and had cytometric features associated with resistance to treatment.[1,5]

Considering the adverse prognostic features, the rapid and marked tumor cytoreduction in 61% of 23 patients confirmed remarkable antitumor activity from a single course of melphalan at a dose only three to four times higher than that given with standard therapy. Responses occurred even in patients who had been refractory to all prior therapies and with plasma cell nucleic acid features associated with drug resistance.[1,5] Thus, as with the VAD regimen, the activity of high-dose melphalan confirmed that primary drug resistance could be overcome by dose escalation.[2]

Although myeloma protein reductions were rapid and marked, the remission durations were short. If one considers tumor-halving time as an index of drug sensitivity, shorter halving times should be associated with longer resmissions. However, we observed the paradox of progressive shortening of both tumor-halving time (medians of 1.5, 0.5, and 0.3 months) and remission duration (medians of 22, 12 and 4 months) from successive treatments (initial chemotherapy, first salvage VAD, and high-dose melphalan, respectively). These findings were compatible with the progressive expansion of more proliferative primordial tumor cells during the disease course.

The high-dose melphalan program was more toxic than any treatment evaluated previously in patients with multiple myeloma. The 31% mortality rate in patients who did not receive bone marrow support was explained by the high incidence of infection during marked and prolonged neutropenia. This complication was alleviated by autologous bone marrow support in older patients who received even higher doses of melphalan. The comparable degree and speed of response after high-dose melphalan with or without marrow support suggested that marrow contamination by well-differentiated tumor cells did not compromise tumor cytoreduction. Persistent thrombocytopenia of 50 to 75,000/μL has been observed in other autologous marrow transplantation programs, but usually in conjunction with considerably higher doses of cytotoxic drugs. Our patients had extensive prior therapy and marrow involvement by tumor cells, either of which might have contributed to the reduced functional capacity of transplanted normal cells.

The short remission duration should discourage further use of this highly toxic treatment in patients with resistant myeloma. The observation by McElwain and Powles of durable remissions in previously untreated patients, however, deserves further exploration.[2,10] We favor such high-dose therapy approach, perhaps in conjunction with total-body irradiation, for consolidation in remission. The availability of autologous marrow stored early in remission would facilitate host support, and selection of patients with chemotherapy-sensitive myeloma should enhance the probability of attaining marked tumor cytoreduction and prolonged disease control. The recent demonstration of common acute lymphoblastic leukemia antigen–positive plasma cell precursors and of plasma cell–specific antigens offers the possibility of in vitro monoclonal antibody treatment for preferential tumor cell removal prior to marrow infusion.[11-13]

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REFERENCES

1. Barlogie B, Smith L, Alexanian R: Effective treatment of advanced multiple myeloma refractory to alkylating agents. New Engl J Med 310:1353–1356, 1984

2. McElwain TJ, Powles RL: High-dose intravenous melphalan for plasma-cell leukaemia and myeloma. Lancet 822–824, 1983

3. Latreille J, Barlogie B, Johnston D, Drewinko B, Alexanian R: Ploidy and proliferative characteristics in monoclonal gammopathies. Blood 59:43–51, 1982

4. Smith L, Barlogie B, Alexanian R: Biclonal and hypodiploid multiple myeloma. Am J Med 1986 (in press)

5. Barlogie B, Alexanian R, Smith L, Dixon D, Smallwood, L, Delasalle K: Prognostic implications of tumor cell DNA and RNA content in multiple myeloma. Blood 66:338–341, 1985

6. Barlogie B, Alexanian R, Gehan E, Smallwood L, Smith T, Drewinko B: Marrow cytometry and prognosis in myeloma. J Clin Invest 72:853–861, 1983

7. McLaughlin P, Alexanian R: Myeloma protein kinetics following chemotherapy. Blood 60:851–855, 1982

8. Alexanian R, Balcerzak S, Bonnet J, Gehan E, Haut A, Hewlett J, Monto R: Prognostic factors in multiple myeloma. Cancer 36:1192–1202, 1975

9. Bonnet J, Alexanian R, Salmon S, Bottomley R, Amare M, Haut A, Dixon D: Vincristine, BCNU, doxorubicin and prednisone (VBAP) combination in the treatment of relapsing or resistant myeloma. Cancer Treat Rep 66:1267–1271, 1982

10. McElwain TJ: Personal communication. 1985

11. Caligaris-Cappio F, Janossy G, Bergui L, Tesio L, Pizzolo G, Malavasi F, Chilosi M, Campana D, van Camp B, Gavosto F: Identification of malignant plasma cell precursors in the bone marrow of multiple myeloma. J Clin Invest 76:1243–1251, 1985

12. Ruiz-Arguelles GJ, Katzmann JA, Greipp PR, Gonchoroff NJ, Garton JP, Kyle RA: Multiple Myeloma: Circulating lymphocytes that express plasma cell antigens. Blood 64:352–356, 1984

13. Anderson KC, Bates MP, Slaughenhoupt B, Schlossman SF, Nadler LM: A monoclonal antibody with reactivity restricted to normal and neoplastic plasma cells. J Immunol 132:3172–3179, 1984

Depletion of Donor Lymphocytes by Counterflow Centrifugation Successfully Prevents Acute Graft-Versus-Host Disease in Matched Allogeneic Marrow Transplantation

By T. de Witte, J. Hoogenhout, B. de Pauw, R. Holdrinet, J. Janssen, J. Wessels,
W. van Daal, T. Hustinx, and C. Haanen

Bone marrow from 22 histocompatible siblings was depleted of 98% of the lymphocytes using a combination of density flotation centrifugation followed by counterflow elutriation. Even with the marrow suppressive influence of methotrexate (MTX), the viability of the hematopoietic stem cells was not affected, as indicated by the normal repopulation after grafting in the evaluable patients. One patient (UPN 9) showed a primary graft failure, possibly resulting from persisting septicemia and long-term antibiotic therapy. Two patients have persistent host lymphocytes, one of whom was examined during relapse; the other remains in remission. Two patients did not receive immunosuppression after bone marrow transplantation (BMT), and acute graft-*v*-host disease (GVHD) developed in both. Nine patients received MTX as immunosuppression following BMT. GVHD did not develop in any of them, but fatal infections in the immediate posttransplant period developed in five patients. Eleven patients received cyclosporine (CsA) after transplantation. Beginning in week 5 after BMT, CsA was gradually replaced by MTX. Acute GVHD, substantial chronic GVHD, or fatal infections did not develop in any of these patients. Removal of 98% of the lymphocytes by counterflow centrifugation prevents development of acute GVHD, provided that immunosuppression is administered after BMT. Graft rejection was not observed, but the number of evaluable patients is limited at present.

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ONE OF THE MAJOR and most frequent complications in allogeneic bone marrow transplantation (BMT) is acute graft-*v*-host disease (GVHD). It occurs in 50% to 70% of all adult recipients despite in vitro matching by HLA analysis and mixed lymphocyte cultures.[1] Based on the postulate that acute GVHD arises from immunologically competent mature lymphocytes present in the graft,[2] several approaches have been investigated to reduce the incidence and severity of GVHD. Methotrexate (MTX),[3] cyclosporine (CsA),[4] and antilymphocyte globulin[5] have been used to suppress the immunocompetent cells after bone marrow infusion, but acute GVHD still may develop in more than 50% of allogeneic matched BMT recipients with an overall fatality of more than 30% or even more if associated fatalities because of interstitial pneumonitis and infections are considered.[6] An alternative to posttransplant immunosuppression is in vitro manipulation of donor marrow to remove the immunocompetent lymphocytes before marrow infusion. Incubation of donor marrow with highly absorbed rabbit antihuman T cell antisera[7] and the use of a murine monoclonal antibody (OKT3) reduced but did not completely prevent acute GVHD.[8] Preliminary data have been presented on 14 patients undergoing transplantation with marrow from matched siblings after depletion of donor T lymphocytes by the use of a cocktail of anti–T cell monoclonal antibodies and rabbit complement. Severe GVHD was prevented, but hematopoietic recovery was slow.[9] Monoclonal antibodies linked to toxins such as ricin have been used to eliminate donor T cells and have been successfully applied to prevent acute GVHD in two children.[10] Lectin separation plus E rosetting gave encouraging results in mismatched BMT in children, but this method is laborious and time consuming.[11]

We previously described a physical method for the separation of human lymphocytes from committed myeloid and erythroid progenitor cells using density gradient centrifugation followed by counterflow centrifugation (CC).[12] CC provides a rapid and reproducible separation technique based mainly on the differences in cell size.[13] Continuous monitoring of the output of the elutriator for cell number and cell size using a light scatter device made this separation method highly efficient and reproducible.[14] Preliminary data showed that the bone marrow repopulation capacity was not impaired.[15] This study presents an improved CC procedure and the clinical results of lymphocyte depletion from human marrow grafts by CC in 22 transplant patients.

MATERIALS AND METHODS

Patient characteristics. All consecutive patients aged <40 years with acute leukemia in the first or second remission (CR) or chronic myeloid leukemia (CML) in the first chronic phase (CP) or accelerated phase (AP) were included in this study (Table 1). Bone marrow remission (defined as less than 5% leukemic blasts in the bone marrow) was confirmed in all patients with acute leukemia.

Transplant procedure. Informed consent was obtained from all recipients and bone marrow donors. Donors were HLA-A, -B, -C, and -D identical siblings. Mutual nonreactivity in mixed lymphocyte cultures was demonstrated in all cases. The transplant conditioning consisted of cyclophosphamide, 60 mg/kg/d (days −6 and −5), followed by 6-mercaptopurinethanesulfonate (Mesna), 28 mg/kg, administered 0, 3, 6, and 9 hours after cyclophosphamide infusion to prevent hemorrhagic cystitis. Fractionated total-body irradiation (TBI) was administered in two equal fractions of 450 cGy each on two consecutive days (day −2, and −1) using an 18-MV photon beam linear accelerator (Saturne, CGR, Buc, France). The midline dose rate ranged between 5.0 to 5.6 cGy/min. Lung and eye shielding was used on the second day of irradiation using individually adapted lead blocks. The corrected lung dose varied from 740 to 810 cGy (Department of Radiotherapy; head, Professor W. van

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Table 1. Patient Characteristics and Disease Status

UPN	Age/Sex	Diagnosis	Date of Diagnosis (mo/yr)	Date of BMT (mo/yr)
1	29/F	M2 (CR1)	10/80	5/81
2	39/M	M4 (CR1)	7/81	1/82
3	29/M	M1 (CR1)	8/81	3/82
4	32/F	M2 (CR1)	7/81	5/82
5	29/M	M4 (CR1)	2/82	9/82
6	18/F	M4 (CR1)	5/82	10/82
7	33/M	M2 (CR1)	5/82	11/82
8	31/M	M2 (CR1)	11/82	3/83
9	36/M	c-ALL (CR1)	10/82	5/83
10	22/F	Null-ALL (CR1)	1/83	6/83
11	38/M	M3 (CR2)	2/73*	8/83
12	27/F	M2 (CR1)	4/83	9/83
13	27/F	M4 (CR1)	8/83	11/83
14	33/M	M2 (CR1)	8/83	12/83
15	22/F	ALL (CR2)	6/77	1/84
16	21/M	CML (AP)	8/83	1/84
17	27/F	M2 (CR1)	11/83	2/84
18	39/M	CML (CP)	11/78	3/84
21	16/M	CML (AP)	11/80	5/84
25	37/M	CML (AP)	12/83	8/84
26	32/F	CML (CP)	7/79	9/84
27	39/M	CML (AP)	8/80	10/84

Abbreviations: UPN, unique patient number; F, female; M, male; M1-4, acute myeloid leukemia, classified according to the French-American-British criteria; c-ALL, common acute lymphoblastic leukemia; Null-ALL: non-T, non-B-ALL.

*This patient had a first CR of 9 years; he received a transplant 9 months after relapse.

Daal). Patients (UPN 15, 21, 25) who were considered to be at a high risk of relapse after transplantation received additional cytarabine ($2 \times 3g/m^2$ on days −3 and −4). Bone marrow was infused 24 hours after completion of the TBI. Sterility of the marrow graft was confirmed by bacteriologic studies (Dr H. Muytjens, Department of Microbiology; head, Professor J. van der Veen).

A successful marrow graft was assumed to have taken place indirectly by a return of the peripheral counts. This was further substantiated by means of erythrocyte markers (Dr A. Drente-Schonk, University Blood Transfusion Services; head, Professor V. Kunst). Differences of the erythrocyte markers between donor and recipient were determined before transplantation; blood samples for red cell markers were taken 4, 8, and 12 weeks post-BMT and every 3 months thereafter. All donor-recipient pairs in this study appeared to have differences in the red cell antigens. When only recipient markers were present, a 100% acceptance was assumed to have taken place by complete disappearance of the recipient marker, which invariably happened between 3 to 6 months after transplantation.

Chromosomal analyses were performed on peripheral blood cells cultured according to Arakaki and Sparkes.[16] Preparations were G-banded,[17] Q-banded,[18] and in some cases also C-banded.[19] When the donor and recipient were of different sex, the sex chromosome constitution was used to distinguish between donor and recipient 6 months after transplantation. In the remaining cases, banding polymorphism studies were performed before BMT; in all cases, sufficient differences were found to permit recognition of the origin of the cells after BMT. MTX was used as routine GVHD prophylaxis for 102 days in nine patients as in the Seattle regimen[6] (Table 2). Since GVHD did not develop in the first five patients, we attempted to omit immunoprophylaxis postgrafting in two patients. Acute GVHD developed in both (see Results), and therefore we

Table 2. Repopulation Data and Clinical Details After BMT

UPN	IS	Recovery (d)		Take		GVHD		Survival (d)
		WBC $>10^9$/L	Platelets $>20 \times 10^9$/L	Red Cell Antigens (%)	Chromosomal Analysis	Acute	Chronic	
1	MTX	28	NE	NA	NA	0	NE	45 (*Aspergillus* pneumonia)
2	MTX	19	20	100	13/13*	0	0	1023+
3	MTX	NE	NE	NA	NA	NE	NE	8 (septic shock)
4	MTX	22	24	100	5/5†	0	0	911+
5	MTX	21	NE	NA	NA	0	NE	22 (*Aspergillus* pericarditis)
6	none	20	22	100	5/5†	3	2	267 (relapse)
7	none	16	20	NA	NA	3	NE	59 (CMV pneumonitis)
8	MTX	25	43	NA	3/3	0	0	278 (relapse)
9	MTX	59	59	NA	NA	0	NE	64 (renal failure)
10	MTX	28	34	NA	NA	0	NE	42 (pneumonia)
11	MTX	18	34	100	0/14†	0	0	212 (relapse)
12	CsA	14	12	100	8/8	0	0	397+
13	CsA	12	14	100	4/4	0	1	346 (relapse)
14	CsA	11	11	100	7/7	0	0	344+
15	CsA	19	16	90	0/8†	0	0	321+
16	CsA	11	16	100	NA	0	0	114 (CMV pneumonitis)
17	CsA	12	12	100	7/7†	0	0	273+
18	CsA	12	12	100	14/14	0	1	257+
21	CsA	15	12	100	8/8	0	0	187+
25	CsA	16	18	100	20/20†	0	0	92+
26	CsA	12	18	100	20/20†	0	0	73+
27	CsA	15	21	100	23/23†	0	0	57+

Abbreviations: IS, immunosuppression; NE, not evaluable; NA, not available; CMV, cytomegalovirus.

*Number of donor cells/number of evaluated cells by banding techniques.

†Number of donor cells/number of evaluated cells by x/y analysis.

Table 3. Recovery of Fractions During Loading and Elutriation

Fraction	Loading	Lymphocytic	Transitional	Stem Cell
Experiment 1 (%)				
Nucleated cells	4.6	35.0	4.1	47.9
Lymphocytes	19.1	80.0	0.6	0.3
CFU-GM	0	0	13.5	86.2
Experiment 2 (%)				
Nucleated cells	8.8	7.2	9.4	51.2
Lymphocytes	56.5	35.5	2.8	0
CFU-GM	0	0.9	10.7	72.0

Recovery (percentage in each fraction calculated from total sample) was achieved during rapid (five-minute) introduction (loading) of the samples (experiment 1, 257 × 10^6, and experiment 2, 194 × 10^6 nucleated cells) and rapid elutriation of the pure lymphocytic fractions (ten minutes) until the transitional fractions were reached. The rotor speed was reduced slowly during elutriation of the transitional fractions.

reinstituted immunoprophylaxis. The remaining patients received CsA, 3 mg/kg, by continuous intravenous (IV) infusion on days −1 to +28, followed by CsA, 3 × 3 mg/kg/d, orally, with a gradual tapering off after 6 weeks and a discontinuation after 12 weeks. Weekly IV injections of MTX were administered from week 5 onward, starting with 2.5 mg/m^2 and weekly increments of 2.5 mg/m^2 until a dose of 10 mg/m^2 was reached. MTX treatment was discontinued in week 16 post-BMT. All patients were managed in single rooms with filtered air under positive pressure throughout the transplant period inside the hospital, and all received oral selective gut decontamination according to different protocols, including co-trimoxazole for pneumocystis prophylaxis and oral acyclovir from days −8 to +60 (UPN 13 to 27). The criteria for acute GVHD described by Glucksberg et al[20] were used for grading severity from 0 to 4.

Separation procedures. Bone marrow was harvested from the donors as previously described.[15] A mononuclear cell fraction with a density <1.070 g/mL was prepared in gradients (Percoll, Uppsala, Sweden), as previously described, but the number of gradients was reduced to 12 by using 250-mL bottles (this will be described in a separate paper). CC was performed with the Beckman J2-21C refrigerated centrifuge equipped with a Beckman JE-6 elutriation rotor and a standard separation chamber (Beckman Instruments, Inc, Palo Alto, Calif). The elutriation system was sterilized and assembled as previously described.[15] A constant counterflow rate was obtained by a peristaltic pump (Masterflex, Cole-Palmer Instruments, Chicago). The pump was connected to a pulse-flattening air chamber. Glucose-phosphate buffer, supplemented with 1% (vol/vol) heat-inactivated fetal calf serum was used as the elutriation medium and maintained at 18 °C. The output of the elutriator was continuously sampled by means of a T drain and analyzed for cell numbers and light scatter using the electro-optical unit of the Hemalog D (Technicon Instruments Corp, Tarrytown, NJ). The light scatter signals were accumulated and displayed on a ND-600 multichannel analyzer (Nuclear Data, Schaumberg, Ill.). Specimens of 10 mL, containing 1.0 × 10^9 nucleated low-density cells were introduced into the elutriator rotor by means of an infusor pump. In vitro clonogenic assays of granulocyte/macrophage colony-forming cells (CFU-GM) and erythroid burst-forming cells (BFU-E) and determination of the number of E rosette–positive cells have been described before.[15,21]

RESULTS

CC procedure. Low-density cells from the first 16 donors were fractionated by stepwise increments of the counterflow rate (0.5 to 1 mL/min) at a constant rotor speed of 2,200 rpm. The duration of one separation was 60 to 70 minutes. Four to five additional runs were necessary because of the small size of the separation chamber. Therefore the elutriation procedure of all low-density cells lasted for six to seven hours. To shorten the procedure, the rotor speed was increased to 3,000 rpm. Specimens were introduced (five minutes) into the rotor at a counterflow rate of 7 mL/min, which was left constant. The rotor speed was gradually decreased (ten minutes) with constant monitoring of the effluent for light scatter properties. The lymphocyte population was displayed by one homogeneous peak. The appearance of a shoulder in the scatter histogram or a distinct new peak indicated the elutriation of the first normoblasts.[14] Elutriation of this population coincides with the appearance of the first erythroid and myeloid progenitor cells.[15] Two to three transitional fractions were collected by small decrements of the rotor speed. Then the rotor was stopped and the contents of the chamber were collected and designated stem cell fraction. Two typical experiments are shown in Table 3. No myeloid clonogenic cells were lost during the rapid introduction of the samples and the rapid decrease in the rotor speed during elutriation of the lymphocytes. The stem cell fraction contained almost no lymphocytes. The transitional fractions contained a low number of lymphocytes and a subpopulation of the small clonogenic cells.

Lymphocyte depletion of donor bone marrow. Low-density cells from the last six donors were separated as was described. The duration of the complete separation was three to four hours. Since the composition of the large-cell fraction and the recovery of the clonogenic cells in this fraction were identical to those of the first 16 separations, the results of all 22 separations are presented in Table 4. The percentage of lymphocytes and E rosette–positive cells contaminating this fraction was 2.2% and 2.4%, respectively, of the original number. The mitogenic response of the fraction containing the stem cells was 6% ± 11% and 11% ± 8%, measured with phytohemagglutinin and mixed lymphocyte culture reactivity, respectively, as published before.[12] The recovery of the myeloid and erythroid clonogenic cells in this fraction was

Table 4. Separation of Bone Marrow From 22 Donors by Density Flotation Centrifugation Followed by CC

Fraction		Aspirated Bone Marrow	Low-Density Cells (d < 1.070 g/mL)	Large-Cell	Fraction Recovery (%)
Nucleated cells	× 10^9	24.7 ± 9.2*	5.6 ± 1.3	2.5 ± 0.9	10.4 ± 3.4
Lymphocytes	× 10^9	3.6 ± 1.3	1.5 ± 0.7	0.08 ± 0.05	2.4 ± 2.1
E rosette + cells	× 10^9	2.1 ± 0.9	1.0 ± 0.5	0.04 ± 0.03	2.2 ± 1.3
CFU-GM	× 10^6	10.5 ± 4.8	8.2 ± 3.9	7.1 ± 3.1	82.3 ± 35.2
BFU-E	× 10^6	21.2 ± 14.1	15.2 ± 10.1	13.0 ± 8.4	79.2 ± 48.6

*Mean ± SD

79%. One out of the 22 grafts was possibly contaminated since the bacteriologic cultures taken from the bag after infusion of the graft were positive, although the recipient remained afebrile for more than 1 week after grafting.

Clinical data. The number of nucleated cells present in the graft was 1.8 to 7.8 × 10^7/kg (Table 5). Despite this low number of nucleated cells, the number of CFU-GM was in the normal range, 2.8 to 31.3 × 10^4/kg (Table 5), and the bone marrow repopulation was not impaired even with the influence of methotrexate-GVHD prophylaxis (Table 2). Patient UPN 9 was an exception in this respect. Persistent gram-positive septicemia developed because of an infected subclavian catheter and a large infiltrate at the site of the tunnel. He was given a second graft on day 38, but he ultimately died of multiorgan failure, despite satisfactory bone marrow recovery. In all evaluable patients (Table 2), acceptance of the donor was substantiated either indirectly by a return of peripheral counts or directly by erythrocyte and/or cytogenetic markers. In two patients (UPN 11 and 15) we were not able to document an acceptance of donor lymphoid cells, despite sufficient follow-up; patient 11 had already relapsed at the time of the examination. All 51 cultured lymphocytes were of male donor origin, and nine cells showed varying composite abnormalities. Patient 15 showed persistent host lymphocytes with a normal female karyotype and no signs of relapse. Red cell antigen analysis showed a true erythroid chimerism with 90% donor cells and 10% recipient cells. Late graft rejections were not observed in this study.

Significant acute GVHD developed in both patients who did not receive prophylactic immunosuppression. A generalized CMV reactivation developed in UPN7, and he died of interstitial pneumonitis. The acute GVHD of the other patient responded to a combination of high-dose steroids and antithymocyte globulin. Extensive chronic GVHD of the skin with recurrent opportunistic infections developed, and she ultimately died in relapse. In none of the evaluable patients who received immunosuppression postgrafting did acute GVHD develop. In five patients out of nine receiving MTX, fatal infections developed within 3 months after BMT. This reflects the longer hypoplastic period after grafting as a result of MTX treatment when compared with CsA. The time to recovery to 10^9/L WBC was 26 ± 12 days (SD), and recovery to 20 × 10^9/L platelets without platelet support was 32 ± 13 days when using MTX as GVHD prophylaxis. The time to recovery was 14 ± 3 days and 15 ± 3 days, respectively, when using CsA. A primary CMV infection developed in UPN 16 after BMT with a graft from a CMV-positive donor. Mild chronic GHVD of the skin developed in two patients, which responded promptly to reintroduction of low dosages of CsA. Four patients have relapsed: two patients with M4-AML in first CR, one patient with M2-AML in first CR, and one patient with M3-AML in second remission.

Eleven patients are currently alive and well without any sign of chronic GVHD from day 57+ to 1,023+ after BMT.

DISCUSSION

The morphology of a hematopoietic stem cell (HSC) is that of a relatively small lymphoblastic cell.[22] The diameter of the putative murine HSC, the colony-forming unit–spleen

Table 5. Nucleated Cells (NC), E Rosette–Positive Cells (T Cells) and CFU-GM Present in Donor Marrow Before and After the Separation Procedures per kilogram Body Weight of the Recipient

	Aspirated Bone Marrow			Graft		
UPN	NC (× 10^7/kg)	T Cells (× 10^6/kg)	CFU-GM (× 10^4/kg)	NC (× 10^7/kg)	T Cells (× 10^6/kg)	CFU-GM (× 10^4/kg)
1	24.3	29.0	15.6	3.3	1.1	15.3
2	30.1	21.0	15.3	3.0	0.8	15.9
3	50.3	53.7	19.5	3.1	0.6	12.6
4	28.4	13.2	9.9	2.2	0.4	6.0
5	21.2	44.7	3.3	2.3	0.4	2.8
6	35.0	25.4	6.3	3.4	0.9	9.7
7	43.0	34.8	17.5	3.8	0.8	7.4
8	36.6	49.4	17.9	1.8	0.2	8.2
9	44.3	20.0	13.3	2.7	0.3	7.7
9*	20.5	31.6	11.4	2.2	0.6	9.1
10	47.4	18.1	24.6	4.1	0.5	15.8
11	34.6	24.8	9.3	2.5	1.0	8.0
12	60.2	51.2	33.6	4.5	0.2	18.0
13	52.6	34.0	10.6	7.8	0.4	12.6
14	68.1	19.4	24.9	6.1	1.0	17.3
15	56.2	11.7	27.2	6.8	0.3	31.3
16	29.7	17.3	NE	3.3	0.4	NE
17	27.7	60.4	4.9	4.2	1.5	6.4
18	21.7	15.6	6.8	3.1	0.4	4.8
21	44.4	48.0	32.7	4.9	0.6	15.8
25	20.9	27.6	5.1	3.9	0.4	7.0
26	33.4	20.3	12.7	4.9	0.5	12.9
27	31.4	34.8	14.7	4.0	1.6	8.9

*A second transplant was given on day +34 after BMT 1.

(CFU-S), was estimated to be 7.2 μm, whereas the size of thymocytes was calculated to be 6.0 μm, as determined by a light-activated cell sorter.[23] CC of murine bone marrow showed an average CFU-S volume of 175 μm^3 and a mean lymphocyte volume of 110 μm^3.[24] Also, human lymphocytes have been shown to be smaller than CFU-GM.[25] The human pluripotent hematopoietic clonogenic cell (CFU-GELMM) gives rise in vitro to mature granulocytes, erythrocytes, macrophages, megakaryocytes, and T lymphocytes.[26] It also has a limited self-renewal capacity in vitro,[27] and this makes it a strong candidate for the pluripotent human HSC. We have shown that these CFU-GELMM could be separated from more than 97% of the T lymphocytes.[12] The normal repopulation capacity of these marrow grafts depleted of 98% of the lymphocytes by a separation based upon cell size confirms that the majority of the human HSC is larger than the majority of the lymphocytes. The small size of the standard Beckman elutriator chamber and large number of donor low-density cells made four to five additional runs in the elutriation procedure necessary. By increasing the rotor speed to 3,000 rpm, the duration of one separation could be reduced to 30 minutes without an impairment of the separation profiles and the viability of the stem cells. The duration of density flotation centrifugation followed by counterflow elutriation was seven to eight hours. Recently, we have developed a new rotor containing four standard elutriation chambers. This now allows rapid lymphocyte depletion within two hours.[28]

CC may have potential advantages over other methods of T cell depletion. First, the number of T cells in the graft can be chosen deliberately. Secondly, the small-cell fraction containing the lymphocytes can be manipulated in vitro, for instance, by monoclonal antibodies or immunotoxins directed against GVHD, causing T lymphocytes to spare the antileukemic cytotoxic cells, and then they are given to the recipient. This possibility is under study in our laboratory.

The absolute number of lymphocytes in grafts of several animal species has been shown to be correlated with the incidence of GVHD.[29,30] The severity of acute GVHD is directly proportional to the number of lymphocytes administered, as shown by the inverse relation between survival time and the numbers of lymphocytes added to mismatched marrow in mice.[31] When 98% of the mature T lymphocytes have been removed by counterflow elutriation, acute GVHD, severe chronic GVHD, and graft rejection can probably be prevented, provided that immunosuppression is being used after grafting. However, acute GVHD developed in both patients without immunosuppression. Complete depletion of the immunocompetent T lymphocytes introduces the problem of graft rejection. Mature lymphocytes, apart from inducing acute GVHD, are also quite effective in causing additional immunosuppression and thus facilitate the acceptance of stem cells.[31] Also Deeg et al[32] showed that the addition of thoracic duct lymphocytes to the marrow inoculum successfully abrogates resistance to marrow grafts from unrelated DLA-nonidentical dogs. This has already been confirmed by an increased incidence of graft failures after intensive T lymphocyte depletion using monoclonal antibodies such as Campath 1,[33] CT-2,[34] and cocktails of monoclonal antibodies.[35,36] The intensity of the pretransplant conditioning regimen seemed to play a critical role in the rejection.[32]

CsA does not prevent proliferation of already activated mature lymphocytes. This explains the failure of CsA to prevent acute GVHD after BMT with nondepleted grafts.[4] CsA inhibits the generation of cytotoxic effector T lymphocytes[37] directed against non-HLA antigens present on the cells in the skin and intestinal mucosa.[38] This may explain why CsA can prevent GVHD when it is given after BMT with grafts containing low numbers of committed mature T lymphocytes.

Repopulation after BMT when using MTX as immunosuppression was slow, although within the limits compared with unmanipulated grafts and MTX postgrafting. Repopulation when using CsA as immunosuppression was fast. MTX was introduced in this group of patients 5 weeks post-BMT to limit the duration of CsA administration in view of its side-effects and the possibility of a higher relapse rate, which was reported in one study.[39] Both MTX and CsA have not been proven to reduce the incidence of acute GVHD.[39,40] The combination of both might be more effective, even when using nondepleted marrow. It is not likely that the combination of CsA and MTX, as being used in this study, was responsible for the reduced incidence of acute GVHD since the maximum dose of MTX was not administered before week 9 after BMT.

Engraftment was proven in all patients indirectly by a prompt return of the WBC and platelet counts, with the exception of UPN 9 who needed a second transplant. Red cell antigens studies revealed complete engraftment in all patients with the exception of UPN 15 who showed a persisting host population of 10%. Chromosomal analysis was performed 6 months after BMT; one patient (UPN 11) was in overt relapse and showed persisting male karyotype with multiple chromosomal abnormalities in a minority of the cultured lymphocytes. UPN 15 showed persisting female (= recipient) karyotype without any chromosomal abnormality. She is still in CR after BMT. Recipient hematopoiesis after grafting with unmanipulated marrow has been described and has been associated with manifest or imminent relapse.[41] But on the other hand, there are three reports of several patients who remained relapse free after host cells have been found in the marrow and/or blood.[42-44] Since our patient (UPN 15) showed persisting recipient cells with a normal karyotype, this may not necessarily indicate an unfavorable prognosis.[42,43]

Severe acute GVHD and chronic GVHD have been shown to prevent a relapse after BMT.[45] In three out of the eight patients with AML in first remission who were at risk of a relapse, a recurrence of the disease was observed. Whether this reflects the absence of GVHD or an inadequate conditioning cannot be concluded from this limited number of patients.

NOTE ADDED IN PROOF

Chromosomal analysis of peripheral blood lymphocytes of patient UPN 15 revealed 26 XY (donor) cells and four XX (recipient) cells. She is in continuing remission.

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REFERENCES

1. Storb R, Thomas ED: Human marrow transplantation. Transplantation 28:1, 1979

2. Heidt PJ, Wagemaker G, Knaan-Shanzer S, Van Bekkum DW: Two distinct types of late onset graft-versus-host disease after bone marrow transplantation in lethally irradiated mice. Transplantation 32:263, 1981

3. Thomas ED, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, Lerner KG, Glucksberg H, Buckner CD: Bone marrow transplantation. N Engl J Med 292:832, 1975

4. Powles RL, Clink HM, Spence D, Morgenstern G, Watson JG, Selby PJ, Woods M, Barrett A, Jameson B, Sloane J, Lawles SD, Kay HEM, Lawson D, McElwain TJ, Alexander P: Cyclosporin A to prevent graft-versus-host disease in man after allogeneic bone marrow transplantation. Lancet 1:327, 1980

5. Ramsay N, Kersey JH, Robison LL, McGlave PB, Woods WG, Krivit W, Kim TH, Goldman AI, Nesbit ME: A randomized study of the prevention of acute graft versus host disease. N Engl J Med 306:392, 1982

6. Thomas ED, Buckner CD, Clift RA, et al: Marrow transplantation for acute nonlymphoblastic leukemia in first remission. N Engl J Med 301:597, 1979

7. Rodt H, Kolb HJ, Netzel B, Haus RJ, Wilms K, Gotze CB, Link H, Thierfelder S: Effect of anti T-cell globulin on GvHD in leukaemic patients treated with BMT. Transplant Proc 13:875, 1981

8. Prentice HG, Blacklock HA, Janossy G, Bradstock KF, Skeggs D, Goldstein G, Hoffbrand AV: Use of anti T-cell monoclonal antibody OKT3 to prevent acute graft versus host disease in allogeneic bone marrow transplantation for acute leukaemia. Lancet 1:700, 1982

9. Prentice HG, Blacklock HA, Janossy G, Gilmore M, Price-Jones L, Tidman N, Trejdosiewicz, Skeggs D, Panjwani D, Ball S, Graphakos S, Patterson J, Ivory K, Hoffbrand AV: Depletion of T-lymphocytes in donor marrow prevents significant graft-versus-host-disease in matched allogeneic marrow transplant recipients. Lancet 1:472, 1984

10. Filipovich AH, Vallera DA, Youle RJ, Quinones RR, Neville DM Jr, Kersey JH. Ex-vivo treatment of donor bone marrow with anti–T-cell immunotoxins for prevention of graft versus host disease. Lancet 1:469, 1984

11. Reisner Y, Kapoor N, Kirkpatrick D, Pollack MS, Dupont B, Good RA, O'Reilly RJ: Transplantation for acute leukaemia with HLA-A and B non-identical parenteral marrow cells fractionated with soybean agglutinin and sheep red blood cells. Lancet 2:327, 1981

12. De Witte T, Koekman E, Geestman E, Plas A, Blankenborg G, Weesels J, Haanen C: Separation of immunoreactive lymphocytes from human pluripotent stem cells (CFU-GM) by means of counterflow centrifugation. Blut 48:139, 1984

13. Sanderson RF, Bird K: Cell separation by counterflow centrifugation, in Prescott DM (ed): Methods in Cell Biology, vol 15. Orlando, Fla, Academic, 1977, pp 1–14

14. De Witte T, Plas A, Koekman E, Blankenborg G, Salden M, Wessels J, Haanen C: Cell size monitored counterflow centrifugation of human bone marrow resulting in clonogenic cell fractions substantially depleted of small lymphocytes. J Immunol Methods 65:171, 1983

15. De Witte T, Raymakers R, Plas A, Koekman E, Wessels J, Haanen C: Bone marrow repopulating capacity after transplantation of lymphocyte depleted allogeneic bone marrow using counterflow centrifugation. Transplantation 37:151, 1984

16. Arakaki TD, Sparkes RS: Microtechnique for culturing leucocytes from whole blood. Cytogenetics 2:57, 1963

17. Caspersson T, Zech L, Johansson C, Modest EJ: Identification of human chromosomes by DNA-binding fluorescent agents. Chromosoma 30:215, 1970

18. Sumner AT: A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 75:304, 1972

19. Scheres JMJC: Identification of two robertsonian translocations with a Giemsa banding technique. Humangenetik 15:253, 1972

20. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED: Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. Transplantation 18:295, 1974

21. De Witte T, Plas A, Koekman E, Blankenborg G, Salden M, Wessels J, Haanen C: Separation of human bone marrow by counterflow centrifugation monitored by DNA-flowcytometry. Br J Haematol 58:249, 1984

22. Barr R, Wang-Peng J, Perry S: Hematopoietic stem cells in peripheral blood. Science 190:284, 1975

23. Van den Engh G, Visser J, Trask B: Identification of CFU-S by scatter measurement on a light activated cell sorter, in Baum SJ, Ledney GD (eds): Experimental Hematology Today. New York, Springer-Verlag, 1979, p 114

24. Inoue T, Carsten A, Cronkite V, Kelley J: Separation and concentration of murine hematopoietic stem cells (CFU-S) using a combination of density gradient sedimentation and counterflow centrifugation. Exp Hematol 9:563, 1981

25. Wells J, Opelz G, Cline M: Characterization of functionally distinct lymphoid and myeloid cells from human blood and bone marrow. II. Separation by velocity sedimentation. J Immunol Methods 18:79, 1977

26. Messner H, Izaquire C, Iamal N: Identification of T lymphocytes in human mixed hematopoietic colonies. Blood 58:402, 1981

27. Ash R, Detrick R, Zanjani E: Studies of human pluripotent hematopoietic stem cells (CFU-GEMM) in vitro. Blood 58:309, 1981

28. De Witte T, Plas A, Koekman E, De Pauw B, Wessels J, Haanen C: Prophylaxis of graft versus host disease using lymphocyte depleted marrow followed by short term immunosuppression. Exp Hematol 13:120, 1985 (suppl 13)

29. Kersey JH, Meeuwissen H, Good R: Graft versus host reactions following transplantation of allogeneic hematopoietic cells. Hum Pathol 2:389, 1971

30. Korbling M, Fliedner T, Calvo W: Albumen density gradient purification of canine hematopoietic blood cells (HBSC): longterm allogeneic engraftment without GVH-reaction. Exp Hematol 7:277, 1979

31. Van Bekkum D: The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. Transplantation 2:393, 1964

32. Deeg HJ, Storb R, Weiden PL, et al: Abrogation of resistance and enhancement of DLA-nonidentical unrelated marrow grafts in lethally irradiated dogs by thoracic duct lymphocytes. Blood 53:552, 1979

33. Hows J, Parreira A, Yin J, Goldman J, Gordon-Smith E: T lymphocyte depletion using Campath 1. Exp Hematol 12:412, 1984

34. Champlin R, Jacobs A, Gale RP, Ho W, Selch M, Lenarsky

C, Feig SA: High-dose cytarabine in consolidation chemotherapy or with bone marrow transplantation for patients with acute leukemia: Preliminary results. Semin Oncol 12:190, 1985 (suppl 3)

35. Martin PJ, Hansen JA, Storb R, Thomas ED: A clinical trial of in vitro depletion of T cells in donor marrow for prevention of acute Graft-versus-Host-disease. Transplant Proc 17:486, 1985

36. Patterson J, Prentice HG, Gilmore M, Blacklock H, Brenner MK, Janossy G, Skeggs D, Ivory K, Hoffbrand AV, Apperley J, Goldman J, Burnett A, Alcorn M, Pearson C, McVickers I, Haan I, Reid C, Wardle J, Bacigalupo A, Robertson AG: Analysis of rejection in HLA-matched T-depleted bone marrow transplants. Exp Hematol 13:117, 1985 (suppl 17)

37. Leapman S: Effects of Cyclosporin A on human lymphocytes in culture. J Clin Lab Immunol 1:67, 1978

38. Tsoi M: Cell mediated immunity to nonHLA antigens of the host by donor lymphocytes in patients with chronic graft versus host disease. J Immunol 125:2258, 1980

39. Zwaan FE, Hermans J, Barrett AJ, Speck B: Bone marrow transplantation for acute nonlymphoblastic leukaemia: A survey of the European Group of Bone Marrow Transplantation (EGBMT). Br J Haematol 56:645, 1984

40. Lazarus HM, Coccia PF, Herzig RH, Graham-Pole J, Gross S, Strandjord S, Gordon E, Cheung NV, Warkentin PI, Spitzer RS, Warm SE: Incidence of acute graft versus host disease with and without methotrexate prophylaxis in allogeneic bone marrow transplant patients. Blood 64:215, 1984

41. Lawler SD, Baker MC, Harris H, Morgenstein GR: Cytogenetic studies on recipients of allogeneic bone marrow using the sex chromosomes as markers of cellular origin. Br J Haematol 56:431, 1984

42. Schmitz N, Godde-Salz E, Loffler H: Cytogenetic studies on recipients of allogeneic bone marrow transplants after fractionated total body irradiation. Br J Haematol 60:239, 1985

43. Singer JW, Keating A, Ramberg R, McGuffin R, Sanders JE, Sale G, Fialkow PJ, Thomas ED: Long-term stable hematopoietic chimerism following marrow transplantation for acute lymphoblastic leukemia: A case report with in vitro marrow culture studies. Blood 62:869, 1983

44. Branch DR, Gallagher MT, Forman SJ, Winkler KJ, Petz LD, Blume KG: Endogenous stem cell repopulation resulting in mixed hematopoietic chimerism following total body irradiation and marrow transplantation for acute leukemia. Transplantation 34:226, 1982

45. Weiden P, Sullivan K, Flournoy N, Storb R, Thomas E: Antileukemic effect of graft-versus-host disease. New Engl Med J 304:1529, 1982

Quantitation and Properties of the Active and Latent Plasminogen Activator Inhibitors in Cultures of Human Endothelial Cells

By Eugene G. Levin

Human endothelial cells release two forms of a plasminogen activator–specific inhibitor: an active form that readily binds to and inhibits plasminogen activators and an inactive or latent form that has no anti-activator activity but which can be activated by denaturation. Latent and active forms of plasminogen activator-specific inhibitor were measured in cultures of human umbilical vein endothelial cells. Latent inhibitor was activated by treatment with 1% sodium dodecyl sulfate (SDS), and both forms were assayed by the ^{125}I-fibrin plate method. After 16 hours, the conditioned medium contained 104.6 U/mL latent inhibitor activity and 2.6 U/mL active inhibitor. The level of each form in the culture medium increased with time although the activity associated with the latent form rose more rapidly: the ratio of latent to active inhibitor activity was 12 at four hours (10.3 U/mL v 0.86 U/mL) and reached 56 at 24 hours (155.3 U/mL *v* 2.80 U/mL). Intracellular inhibitor activity was associated with the active form only; no additional inhibitor activity was observed following SDS treatment of cell extracts. A decline in active inhibitor activity occurred during incubation at 37 °C with a 50% reduction in activity occurring in two hours. Treatment of conditioned medium with 10 U/mL thrombin also resulted in a loss of active inhibitor activity. The latent inhibitor, however, was not affected by either of these conditions. The inhibitor activity lost during incubation at 37 °C or thrombin treatment could be regenerated by SDS treatment, suggesting that the loss of the active inhibitor activity represented a conversion of this form to its latent counterpart. Thus, the concentration, stability, and regulation of these two forms of plasminogen activator inhibitor in human endothelial cell cultures differ significantly.

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HUMAN UMBILICAL vein endothelial cells in culture synthesize and release two components of the fibrinolytic system, tissue plasminogen activator (tPA) and an inhibitor specific for tPA and urokinase.[1-4] tPA found in the culture medium of these cells is coupled to this inhibitor through a sodium dodecyl sulfate (SDS) and heat-stable bond.[1,4] The inhibitor is present in excess of the tPA, and the addition of ^{125}I-tPA to the culture medium results in an increase in its molecular weight and a loss of activity.[1,2,4] It has been reported that in addition to an inhibitor that spontaneously binds to and inactivates tPA and urokinase (active inhibitor) there is a second population of inhibitors that has no antiactivator activity in its native state (latent inhibitor).[2,5] This latent population was detected by reverse fibrin autography[3] following SDS–polyacrylamide gel electrophoresis (PAGE) in samples of conditioned medium depleted of active inhibitor. No inhibitor activity was detected if these same samples were applied to reverse fibrin autographs without SDS-PAGE.[2] It was suggested that the latent activity was induced by SDS treatment during electrophoresis and was due to the separation of the inhibitor from a second protein antagonizing its activity or through the denaturation of the latent protein resulting in an active form. These two inhibitors have been reported as being immunochemically related[5,6] and are considered to be the same protein. Thus, studies of the endothelial cell–derived inhibitor must consider both active and latent forms. To accurately measure latent inhibitor activity, a quantitative assay based on the ^{125}I-fibrin plate method has been developed in conjunction with the ability to activate the latent inhibitor population by denaturation.[2,5] This report presents the relative accumulation of these two inhibitors in the culture medium of endothelial cells and the stability of each to incubation at 37 °C and thrombin treatment.

MATERIALS AND METHODS

Cell culture. Endothelial cells were isolated from human umbilical cord veins as previously described[7] and were cultured on 75-cm^2 flasks or 12-well tissue culture dishes (Costar Data Packaging, Cambridge, Mass) and coated with 2 mg/mL calf skin gelatin (Eastman Chemical Products, Inc, Rochester, NY). Primary cultures were used exclusively in this study. Culture medium containing inhibitor was collected from confluent cultures incubated in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) containing 5% NuSerum (Collaborative Research, Waltham Mass) or RPMI alone. Conditioned medium was centrifuged at 13,000 g to remove cell debris and frozen at −70 °C until used.

Cell extracts were prepared by sonication of cells suspended in 0.5 mL phosphate-buffered saline (PBS) for 5 seconds at 30 W (Heat Systems–Ultrasonics, Inc, Plainview, NY). Samples were spun at 35,000 g, and the supernatent was diluted 1:10 with 0.1 mol/L sodium phosphate–5% Triton X-100 buffer (PT buffer) or made 1% SDS followed by a 1:10 dilution in PT buffer.

Assay of fibrinolytic activity and inhibitor activity. Fibrinolytic activity was assayed on ^{125}I-fibrin–coated multiwell tissue culture dishes (24 wells, 16 mm; Costar Data Packaging) containing in 1 mL: 6 μg human plasminogen, 0.1% gelatin, 0.1 mol/L Tris-HCl, pH 8.1, 5% Triton X-100, and tPA as previously described.[8] The rate of fibrinolysis was determined by measuring the amount of radioactivity released from the surface of the dish, and the results were the average of duplicate samples. Inhibitor activity was measured as follows: samples to be assayed for latent inhibitor were made 1% SDS and incubated for ten mintues at 37 °C. Dose-titration experiments indicated that incubation with concentrations of SDS from 0.1% to 1.5% resulted in the appearance of maximum inhibitor activity in 24-hour conditioned medium (data not shown).[5] Incuba-

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tion for ten minutes was also found to be sufficient for the full activation of latent inhibitor with 1% SDS since incubation for longer periods of time up to 30 minutes showed no additional inhibitor activity. Triton X-100 was added to a final concentration of 5% and the samples diluted into PT buffer. Samples to be assayed for active inhibitor were not treated with SDS but made 5% Triton X-100. Appropriate dilutions were made so that 50% inhibition of tPA would occur within the range of sample volumes tested. Increasing volumes of samples (0 to 150 µL) were placed in 0.5-mL centrifuge tubes containing PT buffer. The final volume in each tube was 400 µL. tPA (0.24 IU, expressed as international units defined by the first international standard of tPA of the World Health Organization code 83/517[9]) was added to each tube and the samples mixed and incubated for ten minutes at 37 °C. Duplicate aliquots (200 µL) from each inhibitor-tPA mixture were added to ^{125}I-fibrin–coated wells containing 750 µL assay buffer (0.1 mol/L Tris-HCl, ph 8.1-0.1% gelatin/5% Triton X-100), and 50 µL of plasminogen was added. The assay was allowed to proceed until 40% to 50% of the ^{125}I counts were removed from control wells not containing inhibitor (about one hour). The percentage of residual activity v sample volume was plotted on a linear graph, and the number of inhibitory units per milliliter for each sample was calculated from the volume of sample giving 50% inhibition of tPA activity. Latent inhibitor activity was calculated by subtracting inhibitor activity in nondenatured samples (active) from that measured in SDS-treated (active and latent) samples. One unit of inhibitor activity is that neutralizing one international unit of tPA activity. The culture medium used in these experiments (RPMI 1640 with or without 5% NuSerum) did not affect tPA activity, nor did medium treated with 1% SDS and diluted 1:10 with 5% Triton X-100. Intra-assay variation was less than 10%.

Adsorption of active inhibitor from the samples was carried out by adding the sample to urokinase-Sepharose (prepared with 1 mg benzamidine-agarose–purified urokinase [Winkinase, a gift from Dr G. Murano, Bureau of Biologics, Food and Drug Administration, Bethesda, Md] coupled to 1 mL CNBr-Sepharose [Pharmacia Fine Chemicals, Piscataway, NJ]) and incubating the mixture for 30 minutes at room temperature while mixing. The gel was removed by centrifugation (two minutes at 12,000 g) and the supernatent collected. To determine whether latent inhibitor might be removed by the urokinase, conditioned medium was incubated with urokinase-Sepharose and latent activity measured. No significant loss of latent inhibitor activity was observed.

Human plasmin was a gift from Dr E. Plow (Research Institute of Scripps Clinic). Plasmin inhibition experiments were performed by adding plasmin to increasing volumes of cell extracts and incubating the mixtures for ten minutes at 37 °C. Residual plasmin activity was measured on ^{125}I-fibrin–coated wells in the absence of plasminogen. The amount of plasmin activity used was equal to that generated in the tPA assay in the absence of inhibitor in one hour.

RESULTS

To measure samples containing a range of latent inhibitor activities, appropriate dilutions of each sample must be made. To insure that such dilution does not compromise the accuracy of the assay, samples were diluted before or after SDS denaturation, and the number of inhibitor units calculated to be in the starting material was compared (Table 1). Sample dilutions made either before or after SDS treatment did not significantly affect the final calculated level of inhibitor activity measured by this assay, and all values fell within 7% of the calculated mean. Thus, inhibitor activity in a variety of samples can be measured and compared. Measurement of the levels of active and latent inhibitor in 24-hour endothelial cell conditioned medium is shown in Fig 1. Inhibition of tPA activity by 50% occurred with 13.2 µL of untreated conditioned medium, an amount equal to 4.5 U/mL of inhibitor activity. However, following SDS treatment, only 0.15 µL (calculated from diluted samples, Fig 1) was needed to inhibit tPA activity to the same extent. Thus, treatment with SDS resulted in an increase of inhibitor activity equal to 400.1 U/mL, almost 90 times that of the untreated sample. Time course studies were performed for a 24-hour period and the level of activity of both latent and active forms determined (Table 2). The activity of both forms increased with time, although not in parallel. Latent activity rose more rapidly and exceeded the activity associated with the active form by 12 times at four hours. By 24 hours the ratio of latent to active inhibitor activity had reached 56 times.

Although the vast majority of extracellular inhibitor was

Table 1. Effect of Dilution on the Activation of Latent Inhibitor with 1% SDS

Dilution*	Inhibitor Activity (U/mL)
Before denaturation*	
Neat	115.4
1:2	107.0
1:4	115.4
After denaturation†	
1:10	99.8
1:20	99.8
1:30	107.0
1:40	93.6

*Twenty-hour conditioned medium (RPMI 1640 only) was diluted as indicated with RPMI 1640, treated with 1% SDS, and diluted 1:10 with 0.1 mol/L sodium phosphate-5% Triton X-100, pH 7.2. Samples were assayed in duplicate as described in Materials and Methods and the inhibitor units multiplied by the dilution factor.

†Sixteen-hour conditioned medium was treated with 1% SDS and then diluted as indicated with 0.1 mol/L sodium phosphate-5% Triton X-100, pH 7.2.

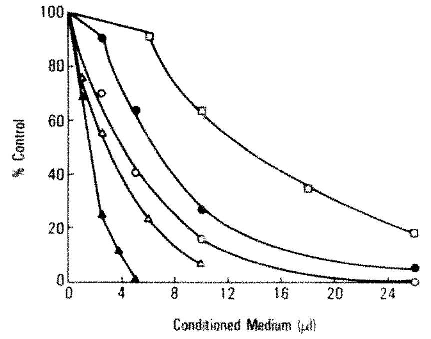

Fig 1. Effect of SDS-treated and -untreated endothelial cell-conditioned medium on tPA activity. Twenty-four-hour conditioned medium was either incubated with 1% SDS for ten minutes at 37 °C and then diluted 1:10 (▲), 1:20 (△), 1:30 (○), or 1:40 (●) with 0.1 mol/L sodium phosphate-5% Triton X-100, pH 7.2 or made 5% with Triton X-100 alone and assayed undiluted (□). Increasing volumes of these samples were assayed for inhibitor activity against 0.24 international units of tPA as described in Materials and Methods. Control samples consisted of tPA alone.

Table 2. Plasminogen Activator Inhibitor Activity in Cultures of Human Endothelial Cells

Source	Inhibitor Activity (U)*		
	Latent	Active	Ratio
Extract†	—	14.9 ± 1.2	
Conditioned Medium‡			
4 h	10.4 ± 0.7	0.86 ± 0.12	12.1
8 h	43.2 ± 7.9	1.40 ± 0.19	30.1
16 h	104.6 ± 15.1	2.60 ± 0.17	40.2
24 h	155.3 ± 45.1	2.80 ± 0.96	55.5

*Samples were treated with either 5% Triton X-100 or 1% SDS followed by 5% Triton X-100 and assayed as described in Materials and Methods. Latent inhibitor activity was calculated by subtracting inhibitor activity in nondenatured samples (active) from that measured in SDS treated (active and latent).

†Extracts were prepared by sonication of ~2 × 10^7 endothelial cells suspended in 0.5 mL PBS and centrifugation at 35,000 g. Before measuring latent inhibitor activity, the active inhibitor was removed by urokinase-Sepharose chromatography as described in Materials and Methods. Data are the mean ± SD of three experiments and represent the total inhibitor activity in the cell extract. —, not detected.

‡Conditioned medium was prepared by incubating cultures in RPMI 1640-5% NuSerum for the indicated period of time and centrifugation at 12,000 g to remove cell debris. Data are the mean ± SD for at least four experiments performed in duplicate and represent inhibitor units per milliliter.

present in the latent form, analysis of the intracellular protein showed the opposite to be true. Cell extract prior to SDS treatment contained 14.9 ± 1.2 U/mL activity, whereas after denaturation, 14.9 ± 0.72 U/mL was present. To eliminate the possibility that any latent inhibitor activity exposed by SDS might be masked by the active form, active inhibitor was removed by adsorption to urokinase-Sepharose and the cell extracts then treated with SDS. No additional inhibitor activity was observed after this treatment (Table 2). Cell extract was also incubated with human plasmin to insure that the inhibitor activity observed was directed against the plasminogen activator and not plasmin. No antiplasmin activity was detected (data not shown). Sonication did not in itself activate the latent inhibitor since sonication of conditioned medium had no effect on the level of latent inhibitor activity.

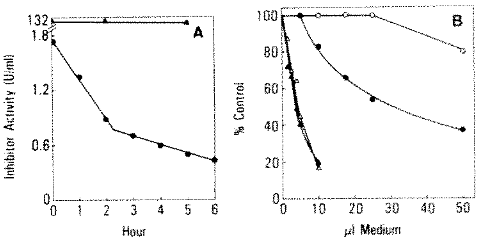

Fig 2. Stability of latent and active plasminogen activator inhibitor activity to incubation at 37 °C and thrombin treatment. (A) Temperature. Conditioned medium was incubated in the absence of cells at 37 °C and aliquots removed at the indicated times. Samples were immediately frozen and then simultaneously assayed for active and latent inhibitor activity as described in Materials and Methods. Key: ●, active inhibitor; ▲, latent inhibitor. (B) Thrombin. Conditioned medium was incubated for two hours at 37 °C in the presence or absence of 10 U/mL thrombin and assayed for latent and active inhibitor activity. Active inhibitor: ●, no thrombin; ○, thrombin. Latent inhibitor: ▲, no thrombin; △, thrombin. Each data point is the average of two experiments performed in duplicate.

This assay was also used to study differences between active and latent inhibitor with respect to stability at 37 °C and thrombin treatment. Incubation at 37 °C leads to a decline in active form activity with a half-life of approximately three hours.[10] In this study, a biphasic rate of inactivation was observed consisting of an initial rapid decline in activity for the first two to three hours ($t_{1/2}$, two hours) followed by a slower loss of activity with a $t_{1/2}$ of about four hours (Fig 2A). In contrast, no significant reduction in latent inhibitor activity could be detected for the same time period. The effect of thrombin on the latent inhibitor activity was also studied. As reported[10] incubation of conditioned medium with 10 U/mL thrombin (9.5 × 10^{-8} mol/L) for two hours at 37 °C resulted in a decline in inhibitor activity when compared with the activity in samples incubated at 37 °C in the absence of the protease (Fig 2B). However, long-term incubation of thrombin with conditioned medium followed by SDS treatment indicated that the latent-form activity was not demonstrably affected by this concentration of thrombin.

To determine whether SDS treatment could activate inhibitor activity lost during incubation at 37 °C or following thrombin treatment, cell extracts were incubated at 37 °C for six hours or treated with 10 U/mL thrombin for two hours at 37 °C, treated with SDS, and assayed for inhibitor activity (Table 3). The use of cell extracts avoided interference by the latent inhibitor activity in the conditioned medium. Incubation for six hours reduced the intracellular inhibitor activity to 29% of control samples left at 4 °C. However, following treatment with SDS, inhibitor activity returned to within 10% of the level in control samples. SDS treatment also resulted in the regeneration of inhibitor activity in thrombin-treated samples; although only 18% of the original activity

Table 3. Inactivation and Regeneration of Active Inhibitor Activity in Cell Extracts

Treatment	Inhibitor Activity (U)
Incubation at 37 °C*	
Triton X-100	7.7
SDS	8.4
37 °C Triton X-100	2.2
37 °C SDS	7.0
Thrombin treatment†	
Triton X-100	5.7
SDS	5.3
Thrombin, Triton X-100	1.0
Thrombin, SDS	4.4

*Extracts were prepared from 2 × 10^7 cells as described in Materials and Methods. Aliquots (100 μL) were incubated at 37 °C for six hours, made either 5% Triton X-100 or 1% SDS followed by 5% Triton X-100, and assayed simultaneously. Control samples were kept at 4 °C. Values are the average of two determinations.

†Extracts were prepared from 3 × 10^7 cells and incubated in the presence or absence of 10 U/mL thrombin for two hours at 37 °C, made either 5% Triton X-100 or 1% SDS followed by 5% Triton X-100, and assayed. Values are the average of two determinations.

remained after incubation with thrombin, 78% was recovered following denaturation with SDS. Thus, activity lost during incubation at 37 °C can be regenerated by SDS denaturation.

DISCUSSION

It has been established that human umbilical vein[1,2,4] and bovine aortic[3] endothelial cells produce a plasminogen activator-specific inhibitor that is present in the culture medium in both active and latent forms.[2,5] Conversion of the latent inhibitor to its active form can be achieved by denaturation of the former with SDS, guanidine hydrochloride, or urea.[2,5] Using this property of activation by SDS in conjunction with the removal of SDS by the formation of micelles with Triton X-100, a method was devised for the quantitative measurement of the latent inhibitor in cultures of human endothelial cells. Based on the ^{125}I-fibrin plate assay, this technique avoids the use of SDS-PAGE and reverse fibrin autography or the need for dialysis to remove the denaturants[5] and enables the simultaneous assay of both latent and active forms. The results from SDS dose titration (unpublished observations) and conditioned medium dilution experiments indicate that at 1% SDS maximum activation of the latent form was achieved. However, it is unclear at this time whether SDS activation is complete under these conditions or whether a portion of the latent inhibitor is refractile to treatment. The regeneration of over 90% of the inhibitor activity lost during incubation at 37 °C (Table 3) suggests that SDS treatment is effective. Purification of the latent form will be necessary to determine whether the optimum specific activity can be attained with SDS treatment. The active inhibitor appears to be stable to short-term incubation with SDS since SDS treatment of cell extracts that contain active inhibitor only (as demonstrated by the complete removal of inhibitor activity by urokinase-Sepharose) did not reduce the activity measured in nondenatured samples. Thus, latent inhibitor activity can be calculated by subtracting inhibitor activity calculated under nondenaturing conditions (active inhibitor) from the activity measured in SDS-treated samples (sum of active and latent inhibitor).

With this assay, the relative levels of active and latent inhibitor were measured in endothelial cell cultures. These results indicate that the inhibitor is primarily in the inactive form in conditioned medium, whereas the intracellular protein is fully active. Accumulation of the two forms in the conditioned medium does not occur in a coordinate manner. The increase in latent inhibitor activity is faster than that of the active material, with a 12-fold excess occurring by four hours and increasing to 56 times by 24 hours. The ratio between latent and active inhibitor was quite variable at 24 hours, with figures ranging from 40 to 140 depending on the cultures tested.

The presence of active inhibitor only in cell extracts, the excess of latent inhibitor in conditioned medium, and the immunochemical similarity between the two human forms[6] suggest that this inhibitor is synthesized and stored in the cell in its active form and then converted to its latent counterpart during or after release from the cell. Judging from the results presented in Fig 2 and Table 3, this conversion occurs spontaneously during incubation at 37 °C. This loss of activity was found to be facilitated by the presence of thrombin. Residual inhibitor activity in samples treated with thrombin for two hours at 37 °C was significantly lower (18%, Table 3) than that in samples incubated for two hours at 37 °C in the absence of thrombin (50%, Fig 2). The regeneration of inhibitor activity by SDS suggests that the loss of activity which occurs during 37 °C incubation or thrombin treatment is not irreversible and that whatever changes occur in the molecule under these conditions can be modified by denaturation to permit the reappearance of inhibitor activity.

The stability of the latent inhibitor in comparison with its active counterpart is potentially of physiologic significance. Inhibitor activity specific for plasminogen activators has been found in plasma[11-15] and in platelets,[16] and it may be involved in tendencies toward thrombotic diseases.[11,12] Changes in the level of inhibitor activity in vivo were demonstrated after infusion with stanozolol.[17] Tissue plasminogen activator in the absence of fibrin has little catalytic activity in terms of plasmin formation[18] but readily binds to the inhibitor.[1,2,4,13] Thus, tPA that is circulating is susceptible to inactivation if free inhibitor is present. A form of the inhibitor that is incapable of interacting with tPA would not compromise the fibrinolytic state of the vasculature. In addition, the presence of the inhibitor in its latent form might also provide stability to proteolytic enzymes. However, the physiologic significance of these properties would depend on whether the latent form is converted to the active inhibitor under the proper circumstances in vivo. Alternatively, it must also be considered that in vivo regeneration of activity does not occur and that the inactivation of the inhibitor is an end point that serves to regulate vascular antiactivator activity. Animal studies with purified material will help clarify the role of this latent form.

REFERENCES

1. Levin EG: Latent tissue plasminogen activator produced by human endothelial cells in culture: Evidence for an enzyme-inhibitor complex. Proc Natl Acad Sci USA 80:6804, 1983

2. Sprengers ED, Verheijen JH, Van Hinsbergh VWM, Emeis JJ: Evidence for the presence of two different fibrinolytic inhibitors in human endothelial cell culture medium. Biochim Biophys Acta 801:163, 1984

3. Loskutoff DJ, Van Mourik JA, Erickson LA, Lawrence D: Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. Proc Natl Acad Sci USA 80:2956, 1983

4. Philips M, Juul AG, Thorsen S: Human endothelial cells produce a plasminogen activator inhibitor and a tissue-type plasminogen activator-inhibitor complex. Biochim Biophys Acta 802:99, 1984

5. Heckman CM, Loskutoff DJ: Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturation. J Biol Chem 260:11581, 1985

6. Sprengers ED, Van Hinsbergh VWM, Jansen BG: Relationship between the fast acting and the latent plasminogen activator inhibitor secreted by human endothelial cells. Thromb Haemost 54:229, 1985

7. Levine JD, Harlan JM, Harker LA, Joseph ML, Counts RB:

Thrombin-mediated release of factor VIII antigen from human umbilical vein endothelial cell in culture. Blood 60:531, 1982

8. Levin EG, Loskutoff DJ: Comparative studies of the fibrinolytic activity of cultured vascular cells. Thromb Res 15:869, 1979

9. Gaffney PJ, Curtis JD: A collaborative study of a proposed international standard for tissue plasminogen activator (tPA). Thromb Haemost 53:134, 1985

10. Van Hinsbergh VWM, Bertina RM, Van Wijngaarden A, Van Tilburg NH, Emeis JJ, Haverkate F: Activated protein C decreases plasminogen activator-inhibitor activity. Blood 65:444, 1985

11. Chmielewska J, Ranby M, Wiman B: Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. Thromb Res 31:427, 1983

12. Juhan-Vague I, Moerman B, De Cock F, Aillaud MF, Collen D: Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. Thromb Res 33:523, 1984

13. Kruithof EKO, Tran-Thang C, Ransijn A, Bachmann R: Demonstration of a fast acting inhibitor of plasminogen activators in human plasma. Blood 68:907, 1984

14. Verheijen JH, Chang GTG, Kluft C: Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb Haemost 51:2141, 1984

15. Wiman B, Chmielewska J, Ranby M: Inactivation of tissue plasminogen activator in plasma. Demonstration of a complex with a new rapid inhibitor. J Biol Chem 259:3644, 1984

16. Erickson LA, Ginsberg MH, Loskutoff DJ: Detection and partial characterization of an inhibitor of plasminogen activator in human platelets. J Clin Invest 74:1465, 1984

17. Verheijen JH, Rijken DC, Chang GTG, Preston FE, Kluft C: Modulation of rapid plasminogen activator inhibitor in plasma by stanozolol. Thromb Haemost 51:396, 1984

18. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem 257:2912, 1982

Differentiation of Cellular Processes Involved in the Induction and Maintenance of Stimulated Neutrophil Adherence

By Denis English and Theodore G. Gabig

Neutrophil adherence stimulated by phorbol myristate acetate (PMA) was investigated by quantitating the attachment of ^{51}Cr-labeled neutrophils to plastic surfaces and to the endothelium of umbilical veins mounted in compartmentalized Lucite chambers. PMA-induced adherence could be functionally separated into an induction phase requiring cellular metabolism and a Mg^{++} dependent maintenance phase that was independent of cellular metabolism. Thus, metabolic inhibitors (*N*-ethylmaleimide, 2-deoxyglucose) blocked adherence when added to neutrophils prior to PMA, but did not cause detachment of cells adhering as a consequence of prior exposure to PMA. PMA failed to induce adherence of neutrophils incubated at low (0.4 °C) temperature, but temperature reduction, even for prolonged periods, did not cause detachment of adherent cells. Thus, the attractive forces that mediate stimulated adherence persist independently of any sustained metabolic response to the inducing stimulus. However, removal of Mg^{++} from the media above adherent cells resulted in immediate detachment, indicating that the cation was required for the persistent expression or maintenance of the attractive forces involved. The extent of stimulated adherence correlated well with the extent of degranulation when rates were varied by limiting the incubation time or stimulus concentration. This correlation was not absolute; in the absence of Mg^{++}, PMA induced degranulation normally but failed to enhance adherence. To explain these findings, we investigated the possibility that PMA-stimulated adherence was maintained by Mg^{++}-dependent cellular adherence molecules released during exocytosis. Supernatants of stimulated neutrophils were devoid of adherence-promoting activity, and only weak activity was recovered in supernatants of mechanically disrupted neutrophils. PMA effectively stimulated the tight adherence of degranulated neutrophil cytoplasts to plastic surfaces and did so in the absence of stimulated granule enzyme release. Thus, conditions have been identified under which degranulation occurs in the absence of adherence (removal of Mg^{++}) and adherence occurs without concurrent degranulation. Since neutrophil cytoplasts do contain some granule products and granule material can be identified on cytoplast membranes, it is possible that degranulation or granule products may be involved in the adherent response. However, hyperadherence was shown to develop in the absence of de novo degranulation. Since similar processes apparently mediate adherence of stimulated cells and cytoplasts, the results indicate that the response is not mediated by cellular adherence molecules released into the surrounding medium during exocytosis.

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UNSTIMULATED NEUTROPHILS display an attraction to other cells,[1] nylon fibers,[2] and glass surfaces[3] in vitro. This adherence is markedly enhanced when neutrophils are exposed to certain metabolic stimuli.[4-9] Both spontaneous and stimulated adherence may play a role in defense against infection and in the inflammatory response. The adhesion of unstimulated cells to the endothelium provides a marginal pool of neutrophils.[10] These readily accessible cells participate in local inflammation or can be quickly recruited to remote inflammatory sites. The ability of chemoattractants to stimulate adherence provides an efficient mechanism for local margination of circulating cells near the inflammatory or infectious stimulus.[6,7] Recent reports have documented the markedly enhanced adherence of neutrophils exposed to agents that initiate oxidative metabolism and degranulation. Enhanced adherence may play a role in the entrapment of infectious agents and neutrophils at sites of inflammation, thereby concentrating cellular metabolites having cytotoxic and microbicidal properties.

The biochemical basis of stimulated adherence has remained elusive. Several lines of evidence implicate substances in neutrophil specific granules as mediators of adherence. Crowley et al demonstrated a profound defect in the adherence of neutrophils from a patient susceptible to bacterial infection.[11] This adherence defect was attributed to the absence of a protein normally found in specific granule membranes.[12] Brockenstedt and Goetzl identified factors that promote neutrophil adherence in supernatants of sonicated neutrophils.[13] Studies of the characteristics and subcellular distribution of these factors implicated acidic proteins (molecular weight, 30,000) from specific granules. Work by Oseas et al has implied a direct role for lactoferrin from neutrophil specific granules in stimulated adherence.[14] Of several agents that stimulate specific granule exocytosis, each was found by Gallin to also enhance neutrophil adherence.[8] In addition, preferential exocytosis of specific granules occurs during the unstimulated (spontaneous) adherence of neutrophils in vitro.[15,16] A similar preferential exocytosis of specific granules was found to occur at sites of experimental inflammatory lesions in vivo.[15] Inhibition of neutrophil adherence by dexamethazone is apparently associated with inhibition of specific granule exocytosis,[17] a finding that supports the proposed involvement of specific granule factors in the enhanced adherence of stimulated neutrophils.

The present study was undertaken to investigate the characteristics of neutrophil adherence induced by phorbol myristate acetate (PMA), an agent that stimulates neutrophil oxidative metabolism and specific granule exocytosis. For these experiments, we quantitated adherence of human neutrophils to plastic surfaces and, using a compartmentalized chamber, to the endothelial wall of human umbilical

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veins. The results demonstrate that induction of adherence by PMA is an active process, susceptible to metabolic inhibitors. In contrast, the adherent state can be maintained passively, with little expenditure of metabolic energy. Experiments were designed to test the hypothesis that a preformed intracellular agent, released by cells upon exposure to PMA, mediates the persistent surface adherence of stimulated neutrophils.

MATERIALS AND METHODS

PMA, zymosan, Ficoll-70, cytochalasin B, *Micrococcus lysodeikticus*, 2-deoxyglucose, *N*-ethylmaleimide, dimethyl sulfoxide (DMSO), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), and Histopaque (Ficoll-Hypaque) were obtained from Sigma Chemical Co, St Louis. Other chemicals and reagents were obtained from Fisher Scientific, Pittsburgh. PMA was dissolved in DMSO at a concentration of 10 mg/mL and further diluted in saline prior to use. The small amount of DMSO carried over to cells with PMA ($<0.002\%$) had no stimulatory or disruptive effect on the adherence of untreated neutrophils to plastic. FMLP and cytochalasin B were dissolved in DMSO at a stock concentration at 10^{-4} mol/L and 5 mg/mL, respectively. Hanks' balanced salt solution (HBSS), obtained without calcium, magnesium and phenol red (GIBCO, Grand Island, NY) was supplemented with 1.0 mmol/L $CaCl_2$, 1.0 mmol/L $MgCl_2$, and 2.5 mmol/L HEPES buffer (pH 7.2), unless stated otherwise. Serum-opsonized zymosan was prepared by the method of Whitin et al,[18] washed with HBSS, and resuspended in HBSS at a concentration of 20 mg/mL. Radiolabeled vitamin B_{12} (^{57}Co-cyanocobalamin, 0.81 μCi/μg) was a generous gift of Dr Bruce Mock, Department of Radiology, Indiana University School of Medicine.

Neutrophil Adherence Assays

Adherence was quantitated by following the attachment of labeled cells to plastic and vascular surfaces. Human neutrophils, isolated from heparinized blood by enhanced sedimentation and centrifugation through Ficoll-Hypaque gradients,[19] were labeled with ^{51}Cr by incubation for 30 minutes with $Na_2\ {}^{51}Cr\ O_4$ (New England Nuclear, Boston, 50 μCi/10^8 neutrophils). Labeled cells were washed twice by centrifugation (500 g, seven minutes) and resuspended in HBSS at a concentration of 3×10^7 neutrophils/mL. To quantitate adherence to plastic, labeled cells, HBSS, and other reagents as listed were added to 12 × 75-mm polystyrene tubes at 37 °C. Unless stated otherwise, 6×10^6 neutrophils were used in a final volume of 400 μL. In preliminary studies, polypropylene, glass, and polystryene tubes were compared for their use in quantitation of neutrophil adherence. Polystyrene tubes were selected since they provided substantially lower values of spontaneous (unstimulated) adherence with no compromise in the extent of PMA-stimulated adherence. After the addition of PMA, labeled cells were incubated for 45 minutes at 37 °C unless stated otherwise. Assay tubes were not shaken, mixed, or otherwise disturbed throughout the incubation period. After incubation, the tubes were vortexed to dislodge loosely adherent cells from the plastic surface. This maneuver substantially decreased the values of spontaneous adherence and facilitated differentiation of the relatively strong surface attachment promoted by PMA. A Fisher Genie vortex was used at a setting of 1 (low). Vortexing was initiated by pressure from the tube and allowed to proceed for approximately 3 seconds. Supernatants were immediately aspirated into a 1-cc syringe through a 25-gauge needle. Careful aspiration left little residual fluid in the assay tubes. The radioactivity that remained with the plastic surface was determined and expressed as the percentage of activity added with the cells. With this procedure, the deviation of replicate determination was consistently less than 4.0% (coefficient of variation) for high-adherence values. The variation was somewhat higher for adherence values of less than 30%. Variability observed from experiment to experiment using neutrophils from different donors was substantially greater. This necessitated inclusion of all appropriate controls in each experiment. Illustrated results reflect the mean and SD of triplicate determinations from one experiment unless stated otherwise. Reported experiments were repeated at least twice to ensure reproducibility.

Neutrophil adherence to vascular walls was assessed by using a compartmentalized Lucite chamber, sections of umbilical veins, and ^{51}Cr-labeled cells. Umbilical cords were obtained immediately after birth of full term neonates. The umbilical vein was flushed thoroughly with HBSS, catheterized, and carefully dissected from the umbilical cord. The vein was opened with a longitudinal cut along the surface and secured to a dissecting board with 25-gauge needles. Circular sections with a diameter of approximately 13 mm were obtained by gently punching through the vein and the underlying matrix with a sharpened no. 6 cork bore. Sections were kept warm (37 °C) in tissue culture medium (TC-199) until used. For adherence experiments, sections were secured in chambers as illustrated in Fig 1. After warming, labeled neutrophils were introduced in the compartment above the endothelial surface of the vein section. For these experiments, 6×10^6 neutrophils were used in a final volume of 200 μL. After the addition of PMA, the chambers were incubated for 45 minutes at 37 °C. Cells that had not become firmly attached to the vessel wall during the incubation period were rinsed out with a moderate jet of 20 ml of HBSS delivered with a 50-ml syringe through a 26-gauge needle. Rinsed sections were placed in tubes to determine their radioactivity, expressed as the fraction of radioactivity added to the chambers. Both spontaneous adherence and the variance of replicate determinations with this technique were greater than that observed with the vortexed test tube assay described above.

To measure weak attractive forces mediated by cellular adherence molecules, the Petri dish assay of adherence described by Brockenstedt and Goetzl[13] was used. Briefly, 6×10^6 labeled neutrophils in 1 mL of HBSS were incubated in plastic 35-mm–diameter Petri dishes with or without 50-μL portions of neutrophil adherence factors prepared as described below. After incubation for 45 minutes at 37 °C, the supernatants were carefully decanted from the dishes and the adherent cells dislodged by the addition of 1 mL of 1.0 mol/L NaOH. Adherence is expressed as the percentage of added radioactivity recovered in the NaOH digests.

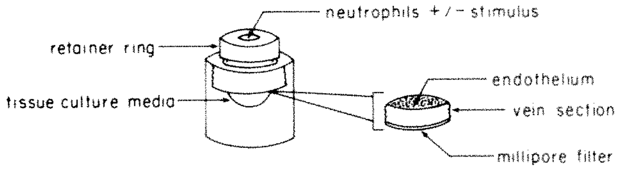

Fig 1. Chamber used to assess neutrophil adherence to the intact endothelium. The chamber is a Lucite blind-well chemotaxis chamber (well diameter, 5/16 in) obtained from Nucleopore, Inc, Pleasanton, Calif. Vein sections were mounted under the upper retainer ring with the endothelium facing up. The lower compartments were filled with tissue culture media, and labeled neutrophils in HBSS were added to the upper compartments. The upper retainer ring prevented the exposure of the cut edges of vein sections to neutrophils. Chambers were warmed in an incubator flushed with 5% CO_2. PMA was then added to the cells. After incubation, the upper compartments were rinsed, vein sections were removed, and adherence was determined radiometrically.

Neutrophil Granule Markers

Neutrophil degranulation was determined with supernatants aspirated from the tubes used for adherence determinations. The supernatants were cooled on ice and centrifuged (750 g, ten minutes) to remove cells. Lysozyme was measured by spectroscopic determination of the lysis of *M lysodeikticus*, as previously described.[20] Results are expressed as the percentage of total lysozyme activity, determined using supernatants of 0.1% Triton X-100–lysed neutrophils.

Neutrophil-associated vitamin B_{12}-binding protein, a specific granule marker,[16] was quantitated using an assay that measures the binding of free *v* protein-bound radiolabeled cyanocobalamin to the surface of albumin-treated charcoal.[21] B_{12}-binding protein was titered in supernatants of stimulated cells and cytoplasts (prepared as described below) and in supernatants of cells and cytoplasts lysed by sonication. Activity is expressed as picograms of vitamin B_{12} bound by supernatants recovered from 4×10^7 neutrophils or cytoplasts. Triton X-100 was not used to increase the efficiency of lysis since the detergent was found to inhibit binding of free vitamin B_{12} to albumin-coated charcoal.

Neutrophil Adherence Factors

A slight modification of the method described by Brockenstedt and Goetzl[13] was used to obtain neutrophil adherence factor (NAF) from disrupted neutrophils. Briefly, proteins were extracted from 3×10^8 neutrophils in a volume of 1.0 mL by sonication followed by acidification. For most experiments, the crude extract was centrifuged (10,000 g, ten minutes) and passed through a Sephadex G-25 gel filtration column 1 cm in diameter and 20 cm long. The Sephadex column was equilibrated and eluted with 100 mmol/L ammonium acetate buffer, pH 6.0. The exclusion volume, determined with high-molecular weight blue dextran, was collected, assayed for protein concentration, and tested without further purification for its influence on neutrophil adherence as described below. Protein concentrations were measured by the method of Bradford[22] with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif).

Experiments were undertaken in an attempt to determine whether adherence-enhancing factors could be detected in supernatants of stimulated neutrophils. The approach used was similar to that described previously for NAF with the exception that we attempted to mobilize the intracellular adherence promoters by metabolic stimulation instead of by sonication. Neutrophils (3×10^8/mL, 1 mL) were incubated with opsonized zymosan (0.5 mg/mL) or PMA (50 ng/mL) for 60 minutes at 37 °C. After stimulation, the cells were removed by centrifugation. Supernatants were assayed for adherence either without further manipulation or after gel filtration through a 1 × 20-cm column of Sephadex G-25 equilibrated either with normal saline or 100 mmol/L ammonium acetate (pH 6.0). Adherence mediated by factors from disrupted or degranulated cells was assessed both in the vortexed tube and Petri dish assays of adherence.

Preparation and Adherence of Neutrophil Cytoplasts

Degranulated neutrophil cytoplasts were prepared by centrifugation of cytochalasin B–treated cells on discontinuous gradients of Ficoll according to the method of Roos et al.[23] The yield of cytoplasts from neutrophils was approximately 80%. Cytoplasts were washed twice and resuspended in HBSS at a concentration of 2.0×10^8 organelles/mL as determined by microscopic enumeration using a hemacytometer. No nucleated cells were observed during microscopic examination. Cytoplasts were incubated with $Na_2\,^{51}CrO_4$ (100 μCi/10^8 cytoplasts) for 30 minutes at room temperature and washed twice prior to assays of stimulated adherence. Adherence was assayed using methods similar to those used for intact neutrophils. Labeled cytoplasts were diluted to a concentration of 1.5×10^7/mL in HBSS containing Mg^{++} and Ca^{++}, and 200-μL aliquots were apportioned into 12 × 75-mm plastic assay tubes. The assay tubes were incubated without disturbance at 37 °C for 45 minutes in the presence or absence of PMA. The tubes were then vortexed as previously described. Supernatants were aspirated, and adherence was determined by an assay of the radioactivity remaining with each tube.

RESULTS

After vortexing, 10% to 25% of the radioactivity of unstimulated neutrophil suspensions remained associated with the plastic surface of the test tubes. When PMA (20 ng/mL) was added, most of the neutrophils (91.8% ± 8.6%, n = 18) became strongly adherent to the plastic surface within 45 minutes. Figure 2 shows how stimulation of adherence is related to the concentration of PMA. Maximal adherence was obtained with >10 ng/mL of PMA. About 50% of the cells remained associated with plastic when 4 ng/mL of PMA was used for stimulation.

Although neutrophil oxidative metabolism stimulated by chemoattractants, fluoride, and other agents requires extracellular Ca^{++}, oxidative responses stimulated by PMA do not.[24] Similarly, neutrophil adherence stimulated by PMA was only slightly impeded in the absence of extracellular Ca^{++}. Adherence was highly dependent on extracellular Mg^{++} (Fig 3); in the absence of extracellular Ca^{++}, PMA induced adherence maximally when the extracellular Mg^{++} concentration was approximately 1.5 mmol/L. PMA did not enhance adherence in the absence of extracellular Mg^{++}, regardless of whether Ca^{++} was present.

Experiments with umbilical vein sections were undertaken to determine whether PMA could induce adherence of neutrophils to biologic surfaces. Although the results obtained using the chamber method were more variable than those obtained with plastic, they were quantitatively similar (Fig 4). Approximately 30% of the radioactivity of unstimulated neutrophils attached to the vessel wall during a 45-minute incubation at 37 °C. This value increased to more than 80% when 20 ng/mL of PMA was added. Intermediate adherence values were obtained with 4 ng/mL of PMA.

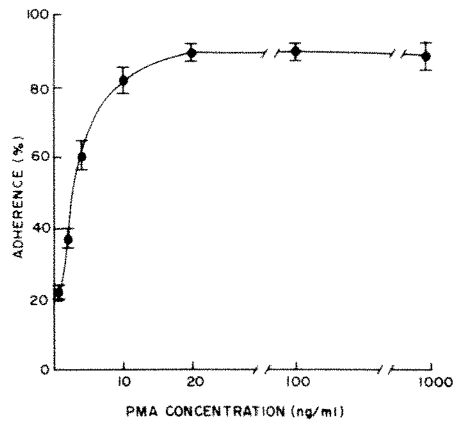

Fig 2. Relationship of stimulated adherence to PMA concentration. Cells in plastic tubes were incubated with PMA for 45 minutes at 37 °C and vortexed. Radioactivity that remained with the plastic after aspiration of the supernatant was determined to calculate the portion of cells that remained adherent.

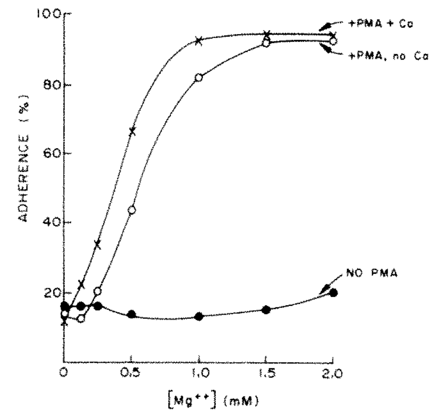

Fig 3. Divalent cation requirements of stimulated adherence. Adherence was determined 45 minutes after the exposure of neutrophils to 20 ng/mL PMA. Where indicated, Ca^{++} was added at a final concentration of 1.0 mmol/L.

Stimulated adherence to the vessel wall was blunted when *N*-ethylmaleimide (1.0 mmol/L) was added prior to PMA. The effect of *N*-ethylmaleimide was irreversible; treatment of neutrophils with the inhibitor at a concentration of 1.0 mmol/L followed by washing resulted in >80% inhibition of adherence (not shown). However, pretreatment of the endothelial surface of the umbilical vein sections followed by rinsing with HBSS did not decrease the subsequent adherence of stimulated neutrophils to the sections (not shown). Exclusion of Mg^{++} or lowering the incubation temperature to 0.4 °C also blunted endothelial adherence (Fig 4). In these respects, the characteristics of neutrophil adherence to plastic and to the endothelial wall of human umbilical veins were similar (see the following material). Being more convenient and less variable, the method with plastic was used in further investigations.

Dissociation of Induction and Maintenance of the Hyperadherent State

Although PMA failed to stimulate neutrophil adherence in the presence of metabolic inhibitors, cells that became adherent to plastic during a previous exposure to PMA at 37 °C did not detach when metabolic inhibitors were added (Fig 5). In addition, although PMA failed to stimulate neutrophil adherence at low temperature, adherent neutrophils did not detach when the assay tubes were immersed in melting ice (Fig 5). In fact, few adherent cells detached when the plastic tubes were held at 0.4 °C for up to two hours (not shown). If near complete inhibition of metabolism was effected by cooling the cells or adding inhibitors, the attractive forces of stimulated adherence apparently persist independently of any sustained metabolic response induced by PMA.

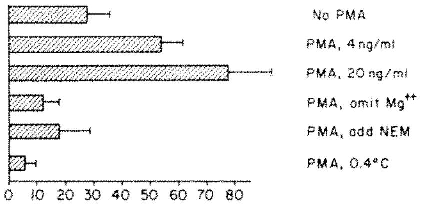

Fig 4. Neutrophil adherence to umbilical vein sections in vein chambers. Chambers were incubated for 45 minutes at 37 °C under the conditions indicated. Results were compiled from three separate experiments performed in duplicate.

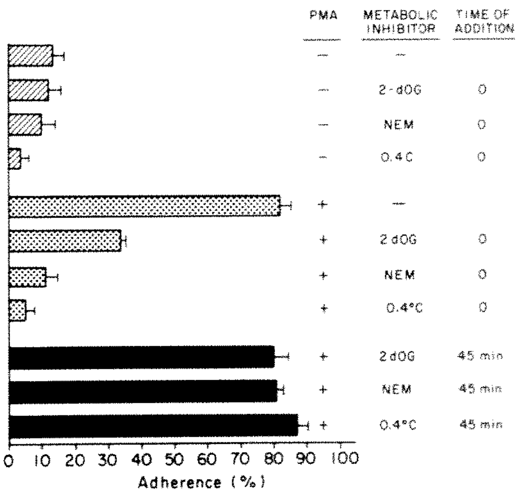

Fig 5. Effect of metabolic inhibition on PMA stimulation of adherence. The inhibitors were added, or the temperature lowered, either prior to the addition of PMA (time 0) or 45 minutes later. Incubation was continued for another 25 minutes and adherence determined. Temperature reductions were effected by immersing assay tubes in a bath of melting ice. *N*-ethylmaleimide (NEM, 1.0 mmol/L), 2-deoxyglucose (2-dOG, 20 mmol/L), and lowered temperature inhibited adherence if added prior to PMA but did not cause detachment of preadherent cells.

Next, experiments were undertaken to determine whether Mg^{++} was necessary for the development (induction) of adherence or, alternatively, whether the cation played an essential role in maintaining the attachment of adherent cells. Adherence was induced by the addition of PMA to neutrophils in media containing Mg^{++}. When the medium above these cells was aspirated and replaced with a Mg^{++}-free buffer, most of the cells rapidly detached (Fig 6). Only

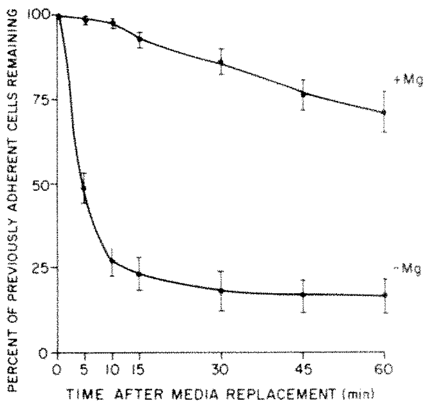

Fig 6. Removal of extracellular Mg^{++} causes detachment of adherent cells. The media above adherent neutrophils was aspirated and replaced with warm HBSS with or without 1.0 mmol/L Mg^{++}. The number of cells that remained adherent thereafter was compared to the number adherent prior to media replacement to determine the kinetics of detachment.

Table 1. Neutrophil Adherence Stimulated by FMLP and Opsonized Zymosan

	Percentage of Adherence at 45 Minutes in Tubes			
Neutrophil Treatment	Without Mg^{++}	With Mg^{++}	With *N*-ethylmaleimide Added at t_0	With *N*-ethylmaleimide Added at t_{30}
None	13.8	16.6	15.1	14.3
Opsonized zymosan (1 mg/mL)	13.4	79.8	13.8	78.2
Cytochalasin B (5 μg/mL)	NT	20.1	NT	NT
FMLP (10^{-6} mol/L)	NT	21.7	NT	NT
FMLP + cytochalasin B	13.1	72.2	13.7	66.0

Abbreviation: NT, not tested.

For determination of adherence, the indicated stimuli were added to ^{51}Cr-labeled neutrophils in polystyrene assay tubes and incubated for 45 minutes. Mg^{++} (1.0 mmol/L) was included in experiments with 1.0 mmol/L *N*-ethylmaleimide. *N*-ethylmaleimide was added either prior to the stimulus (t_0) or 30 minutes later (t_{30}). Values represent the mean of three determinations. For each value, the coefficient of variation (percent SD) was less than 10%.

slight detachment was observed when the replacement buffer was supplemented with 1.0 mmol/L $MgCl_2$, indicating that the experimental manipulations were not sufficient to dislodge the adherent cells. These experiments support the possibility that Mg^{++} exerts its effect in maintenance of adherence.

Neutrophil Adherence Stimulated by FMLP and Opsonized Zymosan

Experiments were then undertaken to determine whether other neutrophil agonists could promote adherence in our assay system in a manner similar to that observed with PMA. Exposure of neutrophils to serum-opsonized zymosan (1 mg/mL) led to persistent surface attachment as detected by the vortexed tube assay method (Table 1). In these experiments, after the suspension of zymosan particles was quickly mixed with neutrophils, the assay tubes were incubated without further agitation or disruption for 45 minutes. When neutrophils were mixed with opsonized zymosan in the absence of Mg^{++}, no stimulation of adherence was observed. Furthermore, removal of Mg^{++} from the media above the adherent cells resulted in surface detachment (not shown). *N*-ethylmaleimide completely inhibited opsonized zymosan–induced adherence when added before the opsonized zymosan but did not cause detachment of cells that were adherent as a result of a previous exposure to the stimulus (Table 1).

FMLP, at a concentration of 10^{-6} mol/L, did not induce the persistent surface attachment of neutrophils necessary for the detection of adherence in our assay system (Table 1). Cytochalasin B, an agent that greatly potentiates neutrophil responses to chemoattractants,[19] also was without effect when tested alone. However, the two agents together induced neutrophil adherence with an intensity nearly equal to that induced by opsonized zymosan and PMA (Table 1). Like adherence induced by opsonized zymosan and PMA, the response to FMLP + cytochalasin B was inhibited when Mg^{++} was removed from the extracellular milieu. *N*-ethylmaleimide inhibited adherence when added to neutrophils prior to FMLP + cytochalasin B but did not cause detachment when added to adhering cells. The aforementioned experiments demonstrate that our data dissociating cellular mechanisms involved in the induction and maintenance of stimulated adherence are not unique to PMA but pertain also to neutrophil surface attachment induced by other, more physiologic agonists of neutrophil metabolism.

On the relationship of PMA-stimulated adherence and degranulation. A strong correlation between adherence and exocytosis was apparent when the rates of both responses were altered by varying the incubation time and PMA concentration (Fig 7). In these experiments, adherence and degranulation were quantitated simultaneously. As a measure of degranulation, the enzymatic activity of lysozyme was quantitated in supernatants aspirated from each plastic tube, whereas ^{51}Cr remaining in the bottom of the tube was measured to quantitate adherence. The correlation of lysozyme release and adherence held when metabolic inhibitors were added or when the incubation temperature was reduced prior to the addition of PMA. Thus, 25.0 mmol/L 2-deoxyglucose, 1.0 mmol/L *N*-ethylmaleimide, and ice bath immersion decreased lysozyme release effected by 20 ng/mL PMA to 19.0%, 7.8%, and 3.1% of control values, respectively. On the other hand, release of lysozyme was not affected by removal of Mg^{++}, a procedure that completely prevented PMA from enhancing surface attachment.

Role of stable cellular mediators. To explain the aforementioned results, we expanded the hypothesis examined by

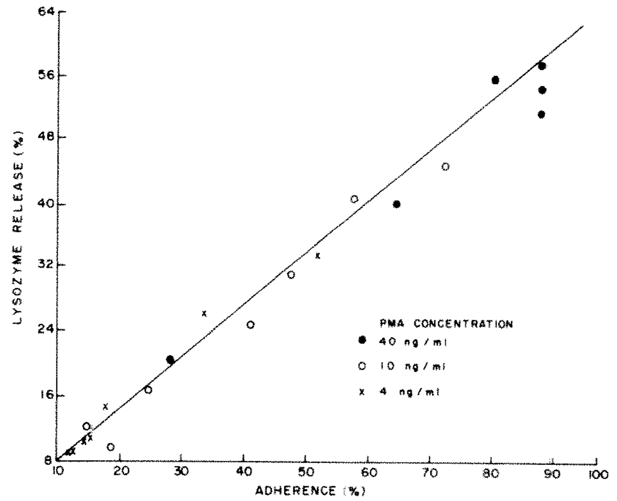

Fig 7. Correlation of neutrophil degranulation and adherence implies a close relationship between the two processes. Lysozyme release was measured in supernatants from tubes used to make adherence determinations. Reaction rates were varied by using different concentrations of stimulus and by varying the incubation time. Each data point is the mean of triplicate determinations from a single experiment.

others[12-14,25-29] that adherence is mediated by one or several intracellular agents that are mobilized, released, or activated during degranulation. Release of essential mediators during exocytosis would be disrupted by metabolic inhibition but not necessarily by removal of Mg^{++}. However, once released, the ability of cellular mediators to promote adherence would predictably require exogenous Mg^{++}. However, we consistently failed to detect adherence-promoting activity in supernatants of PMA-stimulated neutrophils (not shown). Additionally, no activity was released when opsonized zymosan (0.5 mg/mL) was used in place of PMA. In these experiments, a number of conditions were used in an attempt to identify adherence mediators in supernatants of degranulated cells. These included degranulation of cells in the presence and absence of Mg^{++}, assay of supernatants directly or after gel filtration, and use of both the vortexed tube and Petri dish assay of adherence. Under all conditions examined, no detectable adherence-promoting activity appeared in supernatants of human neutrophils upon metabolic stimulation.

Brockenstedt and Goetzl have identified adherence-promoting molecules in supernatants of mechanically disrupted neutrophils.[13] Experiments were designed to characterize this activity in relation to PMA-stimulated adherence. Gel-filtered supernatants of sonicated neutrophils promoted the attachment of fresh neutrophils in the Petri dish assay of adherence, and this activity was, in fact, Mg^{++} dependent (Fig 8). However, this activity did not effect attachment of fresh neutrophils in the vortexed tube assay of adherence (not shown). Therefore, the involvement of this intracellular activity in the response of stimulated neutrophil adherence is questionable.

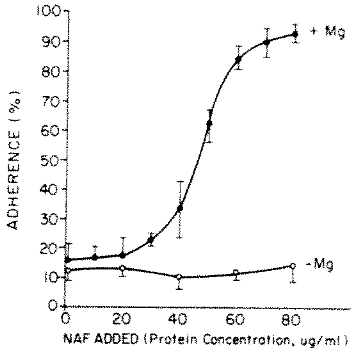

Fig 8. Induction of adherence by intracellular NAF requires extracellular Mg^{++}. NAF was obtained after gel filtration of supernatants of sonicated neutrophils. Fifty microliters of NAF diluted to the indicated protein concentrations were incubated with 950 μL of labeled neutrophils at 37 °C for 60 minutes and tested in the Petri dish assay of adherence. Maximal adherence was obtained with a final protein concentration of about 10 μg/mL (80 μg/mL in the NAF fraction added).

Adherence of neutrophil cytoplasts. To further examine the role of intracellular factors, we investigated the adherence and enzyme release of stimulated neutrophil cytoplasts. The cytoplasts used in this study retained 6.1% ± 1.55% of the lysozyme and 5.4% ± 1.3% of the vitamin B_{12}–binding activity of the cells used to prepare them (n = 3). No stimulated release of this activity was detected when the cytoplasts were suspended in HBSS at a relatively high concentration (2×10^8 organelles/mL) and exposed to PMA. As shown in Table 2, unstimulated cytoplasts released about 10% of their content of lysozyme during a 45-minute incubation at 37 °C, as determined by quantitation of enzyme levels in both supernatants and pellets of the organelles after centrifugation. Slightly less activity was released by PMA-treated cytoplasts. This result was not due to consumption or inactivation of released lysozyme by stimulated cytoplasts since the total lysozyme activity recovered in stimulated and unstimulated reaction mixtures was similar. Table 3 also demonstrates that unstimulated cytoplasts release 24% of their vitamin B_{12}–binding activity when incubated for 45 minutes at 37 °C. When cytoplasts were stimulated with PMA, no further release of this specific granule marker occurred. However, using conditions under which PMA failed to induce release of either lysozyme or vitamin B_{12}–binding protein from cytoplasts, the agonist did promote surface adherence of labeled cytoplasts (Table 2). Approximately 10% of the unstimulated cytoplasts remained attached to plastic assay tubes after vortexing. In the presence of PMA, adherence values approached 40%. These experiments do not exclude the involvement of degranulation or granule products in the adherent response. They do illustrate, however, that adherence can be induced in the absence of concurrent degranulation.

DISCUSSION

The experiments of this report were undertaken to clarify molecular and cellular processes involved in stimulated neutrophil adherence. Our data may clarify the unique metabolic and cationic requirements of adherence relative to degranulation. As noted by O'Flaherty et al in studies of PMA-induced neutrophil aggregation,[9] adherence required extracellular Mg^{++} (but not Ca^{++}) and was disrupted by metabolic inhibition. The present study demonstrates that

Table 2. Lysozyme Release and Adherence of Neutrophil Cytoplasts

Cell Treatment	Lysozyme Activity (Units) in Supernatants	Pellets	Total	Lysozyme Release (%)	Cytoplast Adherence (%)
No stimulation	264 ± 12	2,170 ± 125	2,424	10.89	11.5 ± 1.9
PMA, 50ng/mL	180 ± 6	2,240 ± 136	2,420	7.44	35.4 ± 3.1
PMA, 500ng/mL	132 ± 13	2,080 ± 140	2,212	6.35	42.0 ± 6.4

For determination of lysozyme activity, 4×10^7 cytoplasts (200 μL) were incubated with or without PMA for 45 minutes and centrifuged. Lysozyme levels were assayed in supernatants (released activity) and in 0.2% Triton X-100–treated pellets. One unit of activity caused a decrease of absorbancy at 450 nm of 0.001/min of a suspension of *M lysodeikticus*. Adherence was determined simultaneously with ^{51}Cr-labeled cytoplasts. Results represent the mean and SD of three determinations.

Table 3. Release of Vitamin B_{12}-Binding Protein by Neutrophil Cytoplasts

Cell Treatment	Vitamin B_{12}-Binding Capacity (pg) in Supernatants	Pellets	Total	B_{12}-Binding Protein Released (%)
No Stimulation	234 ± 21	739 ± 43	973	24.0
PMA, 50ng/mL	226 ± 18	630 ± 88	906	24.9
PMA, 500ng/mL	228 ± 27	726 ± 61	954	23.9

Vitamin B_{12}-binding capacity was measured in supernatants and in sonicated pellets. Levels are expressed as picograms of radiolabeled vitamin B_{12} bound/4 × 10^7 cytoplasts.

adherence persists, however, when the metabolism of attached cells is inhibited. Most strikingly, prolonged exposure of adhering neutrophils to ice bath temperatures failed to cause detachment. This result, along with similar data obtained with metabolic inhibitors, permit two informative conclusions. First, it is apparent that stimulated adherence can persist under conditions that totally prevent its induction in cells exposed to PMA. Second, the attractive forces that mediate surface attachment must persist independently of any sustained metabolic response induced by PMA. The former deduction led us to differentiate processes involved in the induction and maintenance of adherence and to consider the biochemistry of the two phases separately. The latter conclusion provides a better definition of the nature of the attractive forces involved and may facilitate the design and interpretation of future investigations. Although the requirement for extracellular Mg^{++} in PMA-stimulated neutrophil adherence has previously been documented,[9] the mechanism by which the cation exerts its effect is completely unknown. Unlike the results obtained with metabolic inhibition, we found that adhering cells detached rapidly when Mg^{++} was removed, indicating that Mg^{++} is necessary for maintenance of the adherent state. Mg^{++} seems to be directly involved in surface attachment and may mediate or provide essential ionic attractions. The possibility that Mg^{++} also plays a role in the development or induction of adherence cannot be excluded by our data.

The assay method developed for this study was designed to differentiate stimulated adherence from the weaker response of spontaneous adherence, the more transient response associated with chemotactic factor–enhanced adherence and from the response of neutrophil aggregation. The latter response proceeds under conditions similar to those of adherence. It is likely that adherence and aggregation are manifestations of the same cellular response to agonists such as PMA. It is not likely, however, that cell–cell attachment or aggregation contributed substantially to the radioactivity we detected in our adherence assay tubes. First, in preliminary experiments, we noted that very little cell–cell aggregation occurs in unstirred cell suspensions, such as those used in our adherence assay. Second, when we examined the inside surfaces of the bottom of the assay tubes with an inverted phase microscope, a finely formed cellular monolayer without cell aggregates was consistently observed. Finally, we suspect that any cell aggregates that settled to the glass surfaces during the incubation period would have been quickly resuspended when the assay tubes were vortexed. Resuspended aggregates would have been eliminated when the fluid above the adherence cells was aspirated. That aggregation possibly results from the same cellular response that promotes surface attachment does not lessen the utility or importance of our adherence assay. It would have been technically difficult, if not impossible, to define the role of cellular metabolism and Mg^{++} in the induction and maintenance of adherence using the technique of leukoaggregation instead of a direct assay of surface attachment.

The effects of *N*-ethylmaleimide indicate that different cellular responses may underlie stimulated adherence and the spontaneous or background adherence of unstimulated cells. In a study of spontaneous human neutrophil adherence to calf endothelial cells, Hoover et al found that *N*-ethylmaleimide exerted only a slight inhibitory effect when preincubated with neutrophils.[1] Substantially more inhibition of spontaneous adherence was observed when the endothelial cells were preexposed to the inhibitor. Similarly, we found that *N*-ethylmaleimide, as well as 2-deoxyglucose, had only a modest influence on the adherence of unstimulated cells to polystyrene tubes (Fig 5). Stimulated adherence was completely prevented by the inhibitor. The effect of *N*-ethylmaleimide on stimulated adherence did not seem to be a result of its influence on the endothelium since preincubation of umbilical vein sections with the drug did not decrease subsequent neutrophil attachment.

The extent of neutrophil degranulation, assessed by quantitating lysozyme release, correlates well with the extent of stimulated adherence when the degree of both responses was varied by altering the PMA concentration or incubation time (Fig 7). Although these results support the implication that adherence is closely related to degranulation, no firm conclusion as to causality can presently be drawn. However, with respect to PMA-induced adherence, the data suggest a hypothesis in which degranulation and adherence are regarded as related but independent events. If adherence is mediated by intracellular factors released during exocytosis, adherence-promoting factors should be detectable in supernatants of disrupted cells. As shown in Fig 8, high–molecular weight fractions of supernatants of sonicated neutrophils were effective in promoting adherence of fresh cells, confirming the observations of Brockenstedt and Goetzl.[13] If similar factors are involved in the maintenance of PMA-stimulated adherence, they then would predictably require Mg^{++} to exert their effect based on assignment of an essential role for Mg^{++} in the maintenance of adherence. As shown in Fig 8, the activity of intracellular adherence-promoting factors is, in fact, Mg^{++} dependent.

The adherence-promoting activity obtained from sonicated neutrophils was not strong enough to withstand the dislodging force used in the vortexed tube adherence assay, suggesting the activity may not be involved in PMA-stimulated adherence. Furthermore, using a variety of experimental conditions, we failed to detect adherence-promoting activity in supernatants of neutrophils stimulated by exposure to either opsonized zymosan or PMA. This result is consistent with the recent observation of Kaplan et al[25] who also described conditions under which neutrophil aggregation precedes enzyme release. The latter result was obtained when a chemotactic factor was used to stimulate the aggregation of neutrophils previously exposed to cytochalasin B. When the kinetics of granular enzyme release and adherence induced by PMA were compared, the results were compatible with the possibility that the latter response depended on the former. However, Kaplan et al were consistently unable to provoke aggregation by exposing neutrophils to supernatants of degranulated cells or to defined constitutents of specific granules such as lactoferrin and lysozyme.[25] Thus, although degranulation and adherence were apparently closely related, the exact relationship of the two responses was not defined.

Indeed, the nature of this relationship is remarkably elusive. Oseas et al[14] provided strong evidence that the specific granule protein lactoferrin mediates adherence of neutrophils stimulated with PMA, but this conclusion has been questioned by several authors including Kaplan et al[25] and Whitin and Cohen[28] who stated that the issue of the involvement of lactoferrin in neutrophil adherent responses has not been settled. Indeed, Boxer et al demonstrated that chemoattractants induced normal aggregation of lactoferrin-deficient neutrophils,[26] and Gallin et al found that specific granule-deficient neutrophils were spontaneously hyperadherent.[27] We feel it is reasonable to conclude that lactoferrin is not directly involved in neutrophil adherence. The larger question pertains to the involvement of any released cellular adherence molecule in the surface attachment of stimulated neutrophils.

Important data pertaining to this question have been obtained in studies with neutrophil cytoplasts. In a previous report, Korchak et al[29] determined that cytoplasts aggregate when exposed to PMA. In the present study, we found that PMA stimulated the surface adherence of cytoplasts in the absence of stimulated lysozomal enzyme release. The cytoplasts used contained low but detectable levels of the granule enzyme markers lysozyme and vitamin B_{12}-binding protein. Thus, it remains possible that granule enzyme products are involved in stimulated adherence. However, if neutrophil and cytoplast adherence proceed by similar mechanisms, the direct involvement of released granular material in surface attachment appears unlikely.

Specific granule exocytosis may enhance adherence by a mechanism that does not directly involve release of a cellular mediator. Molecules involved in the adherent response located on granule membranes may be translocated to the plasma membrane during neutrophil activation. Two specific molecules have been proposed to mediate adherence after translocation, namely, Mo-1[30] and the 60.3 antigen.[31] Recently, Arnaout et al proposed that the anti–Mo-1 and 60.3 monoclonal antibodies recognize one or more epitopes of the same family of surface glycoproteins.[32] The mechanism by which these glycoproteins promote surface attachment is not clear. As glycoproteins, they may possess sticky qualities and play a direct role in adherence, or alternatively, act in concert with other intrinsic membrane alterations or released mediators. Studies comparing the MO-1 and 60.3 antigen composition of stimulated and unstimulated cytoplast membranes may clarify the contribution of these molecules to stimulated adherence and the mechanism by which they exert their effect.

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REFERENCES

1. Hoover RL, Briggs RT, Karnovsky MJ: The adhesive interaction between polymorphonuclear leukocytes and endothelial cells in vitro. Cell 14:423, 1978

2. Yanai M, Quie P: Chemiluminescence by polymorphonuclear leukocytes adhering to surfaces. Infect Immun 32:1181, 1981

3. Wright DG, Kauffman JC, Terpstra RG, Graw RG, Deisseroth AB, Gallin JI: Mobilization and exocytosis of specific granules by human neutrophils during adherence to nylon wool in filtration leukapheresis (FL). Blood 42:770, 1980

4. O'Flaherty JT, Kreutzer DL, Ward PA: Chemotactic factor influences on the aggregation, swelling, and foreign surface adhesiveness of human leukocytes. Am J Pathol 90:537, 1978

5. Gallin JI, Wright DG, Schiffman E: Role of secretory events in modulating neutrophils chemotaxis. J Clin Invest 62:1364, 1978

6. Smith CW, Hollers JC, Patrick RA, Hassett C: Motility and adhesiveness in human neutrophils. Effects of chemotactic factors. J Clin Invest 63:221, 1979

7. Fehr J, Dahinden C: Modulating influence of chemotactic factor–induced cell adhesiveness on granulocyte function. J Clin Invest 64:8, 1979

8. Gallin JI: Degranulating stimuli decrease the negative surface charge and increase the adhesiveness of human neutrophils. J Clin Invest 65:298, 1980

9. O'Flaherty JT, DeChatelet LR, McCall CE, Bass DA: Neutrophil aggregation: Evidence for a different mechanism of action by phorbol myristate acetate. Proc Soc Exp Biol Med 165:225, 1980

10. Lichtman MA, Weed RI: Alteration of the cell periphery during granulocyte maturation: Relationship to cell function. Blood 39:301, 1972

11. Crowley CA, Curnutte JT, Rosin RE, Andre-Schwartz J, Gallin JI, Klemper M, Synderman R, Southwick FS, Stossel TP, Babior BM: An inherited abnormality of neutrophil adhesion. Its genetic transmission and its association with a missing protein. N Engl J Med 302:1163, 1980

12. Babior BM, Crowley CA, Rosin RE, Buchanan MR, Grumbrone MA: GP 180 deficiency: An abnormality of specific granules as the cause of a defect in adherence. Blood 58:699, 1981 (abstr)

13. Brockenstedt LK, Goetzl EJ: Constituents of human neutrophils that mediate enhanced adherence to surfaces. Purification and identification as acidic proteins of the specific granules. J Clin Invest 65:1372, 1980

14. Oseas R, Yank Y, Baehner R, Boxer LA: Lactoferrin: A

promoter of polymorphonuclear leukocyte adhesiveness. Blood 57:939, 1981

15. Wright DG, Gallin JI: Secretory responses of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. J Immunol 123:285, 1979

16. Wright DG, Kauffmann JC, Terpstra GK, Graw RG, Deisseroth AB, Gallin JI: Mobilization and exocytosis of specific (secondary) granules by human neutrophils during adherence to nylon wool in filtration leukapheresis (FL). Blood 52:770, 1978

17. Oseas RS, Allen J, Yang H, Baehner RL, Boxer LA: Mechanism of dexamethazone inhibition of chemotactic factor induced granulocyte aggregation. Blood 59:265, 1982

18. Whitin JC, Ryan DH, Cohen HJ: Graded responses of human neutrophils induced by serum-treated zymosan. Blood 66:1182, 1985

19. English D, Roloff JS, Lukens J: Regulation of neutrophil superoxide release by cellular responses to chemotactic peptides. J Immunol 126:165, 1981

20. Wright DG, Malawista SE: The mobilization and extracellular release of granular enzymes from human leukocytes during phagocytosis. J Cell Biol 53:788, 1972

21. Gottlieb C, Law K-S, Wasserman LR, Herbert V: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity. Blood 25:875, 1965

22. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976

23. Roos D, Voetman AA, Meerhof LJ: Functional activity of enucleated human polymorphonuclear leukocytes. J Cell Biol 97:368, 1983

24. Lehmeyer JE, Synderman R, Johnston RB: Stimulation of neutrophil oxidative metabolism by chemotactic peptides. Influence of calcium ion concentration and cytochalasin B and comparison with stimulation by phorbol myristate acetate. Blood 54:35, 1979

25. Kaplan HB, Edelson HS, Friedman R, Weissman G: The roles of degranulation and superoxide anion generation in neutrophil degranulation. Biochim Biophys Acta 721:55, 1982

26. Boxer LA, Coates TD, Haak RA, Wolach JB, Hoffstein S, Baehner RL: Lactoferrin deficiency associated with altered granulocyte function. N Engl J Med 307:404, 1982

27. Gallin JI, Fletcher MP, Seligmann BE, Hoffstein S, Cehrs K, Mounessa N: Human neutrophil-specific granule deficiency: A model to assess the role of neutrophil-specific granules in the evolution of the inflammatory response. Blood 59:1317, 1982

28. Whitin JC, Cohen HJ: Dissociation between aggregation and superoxide production in human granulocytes. J Immunol 134:1206, 1985

29. Korchak HM, Roos D, Giedd KN, Wynkoop EM, Vienne K, Rutherford LE, Buyon JP, Rich AM, Weissmann G: Granulocytes without degranulation: Neutrophil function in granule-depleted cytoplasts. Proc Natl Acad Sci USA 80:4968, 1983

30. Todd RF, Arnaout MA, Rosin RE, Crowley CA, Peters WA, Babior BM: Subcellular localization of the large subunit of Mol (Mol α; formerly gp 110) a surface glycoprotein associated with neutrophil adhesion. J Clin Invest 74:1280, 1984

31. Harlan JM, Killen PD, Senecal FM, Schwartz BR, Yee EK, Taylor RF, Beatty PG, Price TH, Ochs HD: The role of neutrophil membrane glycoprotein GP-150 in neutrophil adherence to endothelium in vitro. Blood 66:167, 1985

32. Arnaout MA, Dana N, Pitt J, Todd RF: Deficiency of two human leukocyte surface membrane glycoproteins (Mol and LFA-1). Fed Proc 44:2664, 1985

Disappearance of Cytogenetic Abnormalities and Clinical Remission During Therapy With 13-Cis-Retinoic Acid in a Patient With Myelodysplastic Syndrome: Inhibition of Growth of the Patient's Malignant Monocytoid Clone

By Janet Abrahm, Emmanuel C. Besa, Martin Hyzinski, Janet Finan, and Peter Nowell

Median survival is as little as 6 months for patients with refractory anemia with excess blasts who demonstrate an abnormal karyotype in the majority of marrow cells. We treated a patient who presented with 29% marrow blasts and 90% abnormal metaphases with 13-cis-retinoic acid. He achieved a complete clinical and cytogenetic remission during therapy. To determine the mechanism of the response, serial studies were done of the effects of 13-cis-retinoic acid and dexamethasone on in vitro growth of his marrow cells. During clinical remission, when the drug was not administered, marrow growth remained significantly depressed. During relapse, the remission growth pattern was replaced by overgrowth of the karyotypically abnormal monocytoid clone. Clonal growth occurred in cultures containing colony-stimulating activity or dexamethasone but was absent in cultures containing concentrations of 13-cis-retinoic acid achieved in vivo. After the drug was reinstituted, a second clinical stabilization developed. Since 13-cis-retinoic acid inhibits normal monocyte colony growth, we postulate that the patient's unusual clinical responses to the drug were due to in vivo growth inhibition of the malignant monocytoid clone.

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MYELODYSPLASTIC SYNDROME is characterized by abnormalities in cell proliferation leading to single or multiple cytopenias. It is a heterogenous group of diseases, encompassing patients with refractory anemia (RA), refractory anemia and excess blasts (RAEB), refractory anemia and excess blasts in transformation (RAEBT), acquired idiopathic sideroblastic anemia (AISA), and chronic myelomonoctyic leukemia (CMML).[1] It is thought to be due to clonal proliferation of an abnormal stem cell[2] and is considered a preleukemic state because approximately 40% of patients progress into acute myelogenous leukemia.[3] The median survival for a patient with excess marrow blasts or with a karyotype abnormality is 6 months.[4]

13-cis-retinoic acid is a stereoisomer of retinoic acid, a normally occurring metabolite of vitamin A (retinol)[5] that is clinically useful in patients with the myelodysplastic syndrome.[6,7] It has been shown to affect normal and abnormal cell proliferation and differentiation in vitro and to prevent the development of radiation and carcinogen-induced tumors in vivo.[8] Drug concentrations that are effective in vitro (10^{-7} to 5×10^{-6} mol/L)[9-17] are achieved in vivo when the drug is administered orally at 3 mg/kg/d or 100 $mg/m^2/d$,[18,19] doses commonly used in the clinical trials.[6,7,11,17]

We report here the detailed studies of one individual who, 3 years ago, was found to have RAEB. An abnormal clone with a t(2;11) chromosome translocation[20] was also identified at that time. He underwent a complete clinical and chromosomal remission during therapy with oral 13-cis-retinoic acid. Several months after administration of the drug was stopped, he relapsed. Administration of the drug was restarted, and he achieved a second clinical stabilization. His marrow growth patterns in vitro suggested that the clinical responses to oral 13-cis-retinoic acid and his prolonged survival were due to inhibition of proliferation of his malignant monocytoid clone.

CASE REPORT

A 61-year-old man with a 2-year history of an underlying arthritis and vasculitis (diagnosed by biopsy specimen of skin rash in 1980) was noted to have anemia, mild thrombocytopenia, and neutropenia with Pelger-Huët abnormality in the neutrophils (Fig 1). He had been treated with corticosteroids on several occasions with clinical improvement of his arthritis and skin rashes but not his hematologic problems. He also gave a history of exposure to various organic solvents and benzene in his work in tire manufacturing.

In Dec 1981, marrow studies revealed in 90% of the metaphases a t(2;11) (p21-22; q22-23) translocation. Therapy was initiated with 19-nor-testosterone decanoate, pyridoxine, and prednisone, but at month 6 because of worsening of his anemia, he was referred to the Medical College of Pennsylvania to enter the clinical trial of 13-cis-retinoic acid therapy. At the time of referral, he required two units of packed red blood cells monthly. His physical examination was unremarkable, and his blood counts after the transfusion revealed a hemoglobin level of 9 g/100 mL, hematocrit value of 25%, and WBC count of 5,200/μL with 69% bands, 28% lymphocytes, and 2% monocytes. His platelet count was 116,000/μL, and the reticulocyte count was 1.5%. His bone marrow was hypercellular (80%) with evidence of dysplasia in all cell lines. An increase in myeloblasts to 21% was observed. He was classified as having RAEB by the French-American-British recommendations.[1] His cytogenetic studies again showed the translocation in 90% of the metaphases, with no other abnormality identified.

All medications except for maintenance doses of dexamethasone (3 mg/d) were discontinued 1 month before starting the clinical trial with 13-cis-retinoic acid. In month 7, 100 $mg/m^2/d$ (230 mg/d) 13-cis-retinoic acid therapy was initiated. At 10 months, the dexamethasone dose was reduced to 0.75 mg/d. Twelve months from his initial presentation (after 5 months of 13-cis-retinoic acid therapy), his blood counts had normalized, and he was transfusion independent. Except for an exacerbation of the rash and arthritis requiring a brief period of increased doses of dexamethasone he did well. By month 15, marrow blasts were decreased to 6%, but 60% of the

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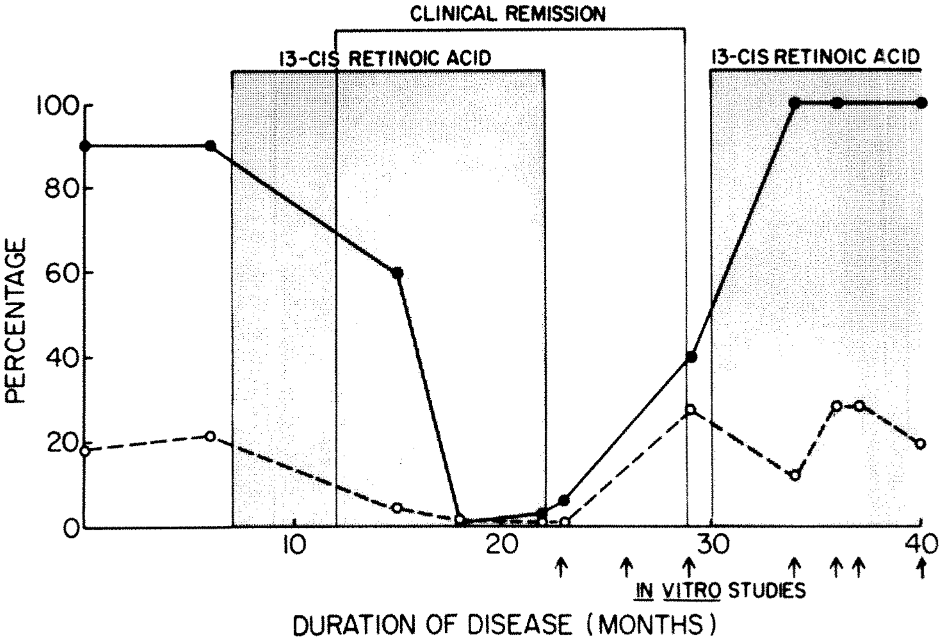

Fig 1. Clinical course of patient J.L. Association of oral 13-cis-retinoic acid therapy with clinical remission. The duration of disease is shown on the abscissa. Key: ●—●, the percentage of abnormal metaphases in marrow cells freshly obtained from the patient; O-----O, the percentage of marrow blasts seen on Wright-Giemsa-stained smears of marrow aspirates; ↑, times when growth of patient marrow cells was studied in vitro (see Fig 3).

marrow metaphases still showed the abnormal clone. At that time the 13-cis-retinoic acid dose was halved because, in spite of changes in his diet, the patient's triglyceride levels had risen to more than 900 mg/dL and his cholesterol had risen above 400 mg/dL. In month 18 his bone marrow studies were normal. At that time, he did not demonstrate any abnormal metaphases, there were no excess marrow blasts, no Pelger-Huët–type cells were apparent, and his peripheral blood counts were normal. At month 22, because of continued clinical remission with fewer than 1% marrow blasts and concern over the long-term effects of the elevated lipids, the 13-cis-retinoic acid was discontinued after 16 months of 13-cis-retinoic acid therapy. One month later, marrow blasts remained <1%, but one of 18 metaphases examined was cytogenetically abnormal with the same t(2;11) translocation that was present at the time of diagnosis.

The patient remained in a clinical remission without receiving therapy until month 29 when the Pelger-Huët abnormality and dysplastic marrow changes returned: 40% of the marrow cells showed the abnormal karyotype, and marrow blasts had risen to 28%. At month 30, the WBC count was 2,400/μL with no normal granulocytes seen; the platelet count was 111,000/μL. 13-cis-retinoic acid therapy, 100 mg/m^2/d (230 mg/d) was restarted, and the dexamethasone continued at 0.75 mg/d. He required six units of packed red blood cells in months 34 and 35 and two units in month 36. In months 34 and 36, 100% of the metaphases were abnormal and the marrow was hypercellular (88%) with marrow blasts of 12% and 29%, respectively. His WBC count had risen to 5,700/μL and platelet count to 133,000.

At month 40, 10 months after 13-cis-retinoic acid therapy had been restarted, however, his marrow cellularity had fallen to 45% with <20% blast forms seen. The t(2;11) translocation was still present in 100% of marrow metaphases. His WBC count was 6,800/μL with 45% bands and no pseudo-Pelger-Huët-type cells; the platelet count was 233,000/μL and the hematocrit value was depressed at 20. At month 42, the 13-cis-retinoic acid therapy was discontinued because abnormal liver function tests had developed.

MATERIALS AND METHODS

Marrow was obtained with informed consent from normal donors (8) and patient J.L. enrolled in the clinical trial of 13-cis-retinoic acid therapy. At each time patient J.L. was studied, marrow from a normal donor was grown under the same culture conditions using the same stimulating materials as were used to study the patient.

Chemicals

Phytohemagglutinin (PHA HA-15, Burroughs-Wellcome, Greenville, NC) was reconstituted in distilled water and stored at −80 °C until used. 13-cis-retinoic acid (kindly provided by Hoffman-La Roche Co, Nutley, NJ) was stored in darkness at −20 °C and dissolved in ethanol for each experiment. Dexamethasone (a gift of Merck, Sharpe and Dohme, Inc, West Point, Pa) was dissolved in 75% ethanol. The final concentration of ethanol in the cultures did not exceed 0.1%.

Stimulating Materials

Media conditioned by normal human peripheral blood leukocytes exposed to 1% vol/vol PHA according to the method of Aye et al[21] served as the source of granulocyte-monocyte colony-stimulating activity (CSA). The media were shown to be most active undiluted. 13-cis-retinoic acid (10^{-7} to 10^{-6} mol/L) and dexamethasone (10^{-7} to 10^{-5} mol/L) were included in the stimulating material alone and in combination in some dishes. These concentrations are those achievable by oral administration of the drugs.[18,22-24]

Culture Techniques

Marrows were placed into semisolid agar cultures as previously described.[25] Briefly, bone marrow aspirates from normal healthy donors or patients were drawn into syringes containing sterile preservative-free heparin, 250 μ/mL (GIBCO, Grand Island, NY). They were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), centrifuged at 1,000 g for 30 minutes, and the interface cells washed and suspended in RPMI 1640 medium supplemented with 15% fetal bovine serum, 20 mmol/L L-glutamine, 1% vitamins, and 1% penicillin/streptomycin (10,000 U/mL) (Flow Laboratories, Inc, McLean, Va). Marrow cells were thus washed free of plasma and drugs contained in the plasma.

Cells were added to a mixture of Iscove's modified Dulbecco's medium (IMDM) (GIBCO) or Dulbecco's modification of Eagle's medium (DMEM) (Flow) modified according to the method of Metcalf[26] with 20% fetal calf serum (Hyclone Tissue Culture

Products, Logan, Utah) and 0.3% Bacto-Agar (Difco Laboratories, Detroit) to a final concentration of 100,000 cells/mL. Thereafter, 1 mL of the cell suspension was placed into 35-μL gridded Petri dishes (Lux Lab-Tek Division, Miles Laboratories, Naperville, Ill) to which 100 μL of stimulating material (see the preceding) had been added. Duplicate or triplicate cultures were done.

After gelling, the cultures were incubated for up to 14 days in either a 5% CO_2 (for IMDM-containing cultures) or a 10% CO_2 (for DMEM-containing cultures) fully humidified atmosphere at 37 °C. They were fixed with formol calcium, transferred to 3 × 2-in glass slides, dried under cellulose acetate filter papers (Schleicher and Shuell, Inc, Keene, NH), and then stained for chloroacetate and alpha naphthyl acetate esterases by the method of Yam et al[27] as modified by Abrahm and Smiley.[25]

Groups of greater than ten cells were considered clusters and groups of greater than 50 cells, colonies. Colonies were considered granulocytic if more than 90% of the cells in the colony contained chloroacetate esterase, monocytic if more than 90% of the colony cells contained alpha naphthyl acetate esterase, and mixed if neither enzyme predominated by 90%.

Methylcellulose stock (2.5%) for methylcellulose culture was prepared by the method of Worton et al[28] except that IMDM was used as the culture medium. Briefly, methylcellulose (Methocel Premium A4M, Dow Chemical Co, Midland, Mich) was dissolved in water at 90 to 100 °C and mixed upon cooling with an equal volume of IMDM prepared at 2× concentration. This mixture was stirred overnight at 4 °C. At the time of culture preparation, methylcellulose stock was mixed with the cell suspension and the appropriate stimulating material at room temperature to a final concentration of 1.2% methylcellulose and 100,000 cells/mL. One milliliter of the mixture was placed in each gridded Petri dish and incubated in a fully humidified 5% CO_2 atmosphere for variable lengths of time. Quadruplicate cultures were performed for each culture condition.

Chromosome Studies

Marrow aspirates. Bone marrow specimens were processed on the day of aspiration and after 24 hours in culture and stained by the Trypsin-Giemsa-banding method as described.[29] For each study, a total of 18 to 38 chromosome counts were done with at least 3 karyotype analyses. Periodically, studies were repeated to determine the effects of therapy.

In vitro culture. On day 8 or 9 of culture, individual plucked colonies or cells from the entire culture dish were washed free of methylcellulose in Hanks' balanced salt solution (GIBCO), placed into hypotonic solution, and further processed by standard methods for chromosome analysis.[30]

Statistical Methods

Student's *t* test for paired samples was used.

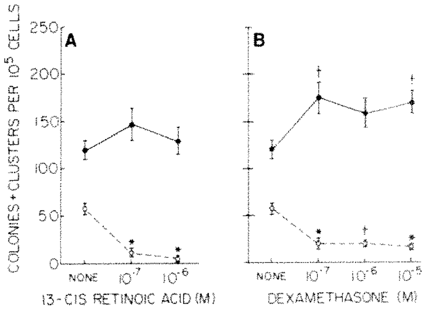

Fig 2. Effect of 13-cis-retinoic acid (left panel) and dexamethasone (right panel) on the growth of GM-CFU from normal donors. Marrow cells from eight donors were cultured, fixed, and histochemically stained as described (Materials and Methods). Key: ●—●, granulocyte colonies + clusters/10^5 cells plated; ○-----○, monocyte colonies + clusters/10^5 cells plated; II, SEM; *, *P* < .005; †, *P* < .025.

RESULTS

Response of Normal Marrow to Incubation With 13-Cis-retinoic Acid and Dexamethasone

Figure 2 illustrates the effect on normal myelopoiesis of 13-cis-retinoic acid and dexamethasone in the range of concentrations achieved by the patient during his therapy. The total number of granulocyte, monocyte, and mixed colonies is not significantly changed by either therapy or by a combination of the two drugs (data not shown).

However, as is shown, 13-cis-retinoic acid or dexamethasone at concentrations of 10^{-7} mol/L or greater significantly decreased the number of monocyte colonies ($P < .005$, except 10^{-6} mol/L dexamethasone, $P < .025$). Dexamethasone (10^{-7} and 10^{-5} mol/L) increased the number of granulocyte colonies ($P < .025$).

In Vitro Studies of Patient J.L.

Marrow from the patient was studied on seven occasions beginning after the completion of his first course of 13-cis-retinoic acid and continuing during the second period of 13-cis-retinoic acid administration (Fig 3). The number of granulocytic and mixed colonies and clusters is shown in the left panel, whereas that of monocytic is shown in the right. He was first studied in month 23 when 13-cis-retinoic acid therapy had been discontinued for 4 weeks. He was in a

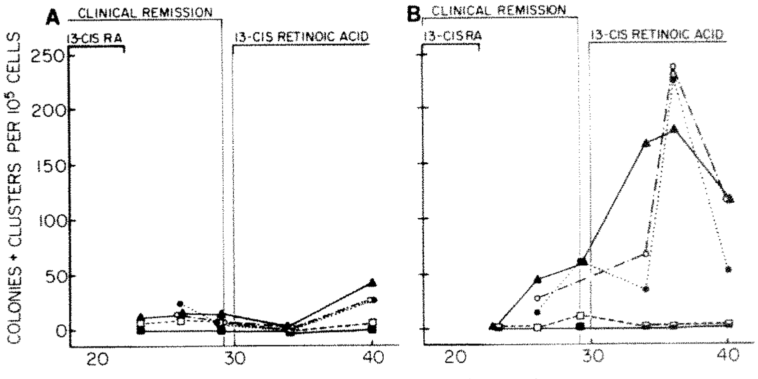

Fig 3. Effect of 13-cis-retinoic acid and dexamethasone on growth of bone marrow GM-CFU from patient J.L. Marrow cells obtained seven times during the course of the patient's disease were cultured, fixed, and histochemically stained as described (Materials and Methods). Data from six cultures are shown. Granulocyte and mixed cluster + colony growth/10^5 cells plated is shown in the left panel, monocyte in the right. Points represent the mean of triplicate cultures. Key: ▲—▲, cultures stimulated only by CSA; ○—·—·—○, those containing 10^{-7} mol/L dexamethasone; ●—●, those containing 10^{-6} mol/L dexamethasone; □-----□, those containing 10^{-7} mol/L 13-cis-retinoic acid; ■—■, those containing 10^{-6} mol/L 13-cis-retinoic acid.

complete clinical remission with no abnormal metaphases found, normal marrow appearance, no Pelger-Huët-type cells seen on blood smear and normal peripheral blood counts. At that time, the only indication that he was not normal was his marrow growth pattern. The number of colonies grown by his marrow cells stimulated by CSA was only 10% of that of normal donor marrows. The proportion of granulocyte to monocyte colonies was similar to that seen in normals, and monocyte colonies were eliminated by 10^{-7} or 10^{-6} mol/L 13-cis-retinoic acid. He was still in clinical remission and not receiving therapy when he was next studied in month 26, but his marrow growth remained abnormal. He was now growing only one third of the number of colonies grown from normal donor marrow, and the proportion of monocyte to granulocyte colonies had become reversed from that seen in normal marrows. Inhibition of monocyte colony growth by dexamethasone was less than that seen in normal marrows, but 13-cis-retinoic acid again completely inhibited monocyte colony growth.

In month 29, after 13-cis-retinoic acid had been discontinued for 7 months, the patient was in relapse. Colony growth stimulated by CSA was similar to that seen in month 26, but at this time, monocyte colony growth was inhibited only by 13-cis-retinoic acid. In month 34, when 100% of the marrow metaphases showed the t(2;11) translocation, granulocyte colonies had disappeared whereas monocyte colony numbers had significantly increased. They were partially inhibited by dexamethasone, completely by 13-cis-retinoic acid. In month 36, dexamethasone no longer inhibited clonal growth, but it was completely inhibited by 13-cis-retinoic acid. Chromosome studies of cells from these monocytoid colonies demonstrated the t(2;11) translocation.

In month 37, no colonies grew under any culture conditions (data not shown). However, in month 40, when the peripheral blood smear had become more normal and the blast percentage and marrow cellularity had decreased, studies showed decreased numbers of monocyte colonies and the reappearance of granulocyte and mixed colonies and clusters.

DISCUSSION

As noted previously in studies by ourselves and others,[9,12,31,32] 13-cis-retinoic acid inhibits monocyte colony growth in marrows from normal donors. In our previous studies we found that 5×10^{-6} or 10^{-5} mol/L 13-cis-retinoic acid increased granulocyte colony numbers to greater than those seen in control cultures.[32] However, in those studies and the current ones, we saw no increase when using less than or equal to 10^{-6} mol/L 13-cis-retinoic acid when cultures were examined at ten or 14 days of incubation. We cannot explain why our studies differ from those that found that 3×10^{-7} or 10^{-6} mol/L 13-cis-retinoic acid increased GM-CFU 100% or 50%, respectively.[9,10,12] Our findings do agree with those of several other investigators.[33-35] Differences in CSA source (Mo cell- or giant cell tumor (GCT)-conditioned medium), in culture techniques, in methods of counting colonies, or in the day of incubation at the time of count may account for the differences observed.

Abnormal growth in vitro, as was demonstrated in the marrow from this patient, has been reported in up to half the patients suffering from myelodysplastic syndrome,[15,35,36] though one study reported equivalent growth.[33] The present report is the first study to show that even when a patient was apparently normal and in a complete clinical and chromosomal remission, the marrow growth pattern remained abnormal.

In addition, this is the first reported RAEB patient with a karyotype abnormality in whom oral administration of 13-cis-retinoic acid was associated with a complete cytogenetic and clinical remission.[20] 13-cis-retinoic acid might have benefited this patient either by inducing differentiation of the malignant clone or by inhibiting its proliferation, allowing normal marrow elements to repopulate.

13-cis-retinoic acid has been shown to induce differentiation in cells from a patient with congenital neutropenia.[37] Irreversible differentiation is also induced in cells of the promyelocytic leukemic cell line, HL-60,[38] and in a minority of cells freshly obtained from patients with acute promyelocytic leukemia.[16,17,39]

Inhibition of proliferation is seen with cells of the KG-1 myeloblastic leukemia cell line,[10] cells from acute lymphocytic leukemia/lymphoma cell lines[34] and cells freshly obtained from some chronic myelogenous leukemia[11,13] and acute nonlymphocytic leukemia patients.[10,34] All-trans-retinoic acid has been shown to inhibit proliferation of the GM-CFU of some patients with myelodysplastic syndrome.[33] In this patient, J.L., in vitro studies demonstrating complete inhibition of proliferation of the abnormal monocytoid clone by concentrations of 13-cis-retinoic acid similar to those he was achieving in vivo suggest that his clinical improvement was due to in vivo inhibition of proliferation of his abnormal clone.

This effect, however, seemed completely reversible when the 13-cis-retinoic acid was removed, either in vivo by discontinuing the drug or in vitro by washing the marrow cells free of the drug before placing them into culture. This reversibility has previously been noted in studies in many other systems in which 13-cis-retinoic acid either inhibits proliferation or prevents carcinogen- or radiation-induced tumor growth.[8] It is likely that renewed clonal proliferation occurred while therapy was discontinued because several months later the same abnormal clone could again be demonstrated in his marrow cells. In vitro studies would have predicted this regrowth since clonal growth was inhibited in the presence of 13-cis-retinoic acid but was abundant in the presence of CSA or dexamethasone.

Continued inhibition of in vivo clonal proliferation by orally administered drug might have accounted for his initial complete remission. Similarly, the current return of his marrow cellularity to normal, the decrease in marrow blasts, the normalization of his platelet counts, and the return of normal granulocytes may be due to such inhibition.

It is of interest that the abnormal clone was monocytoid and that monocyte colony development from normal progenitor cells is inhibited by 13-cis-retinoic acid. Since 13-cis-retinoic acid is effective in only some cases of myelodysplastic syndrome, it may be that clinical responses occur in cases in which a monocytoid clone predominates. Studies of other patients with similar clinical courses and clonal histochemical and chromosomal markers will be required to evaluate this hypothesis further.

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REFERENCES

1. Bennett JM, Catovski D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Salton C: The French-American-British (FAB) Cooperative Group: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

2. Koeffler HP, Golde DW: Human preleukemia: Ann Intern Med 93:347, 1980

3. Mertelsmann R, Moore MAS, Clarkson BD: Sequential marrow culture studies and terminal deoxynucleotidyl transferase activities in myelodysplastic syndromes, in Schmalzl F, Hellreigel KP (eds): Preleukemia. New York, Springer-Verlag, 1979, p 1

4. Coiffier B, Adeleine P, Vialer J, Byron P, Fiere D, Gentilhomme O, Vuvan H: Dysmyelopoietic syndrome: a search for prognostic factors in 193 patients. Cancer 52:83, 1983

5. Frolik CA: Metabolism of retinoids, in Sporn MB, Roberts AB, Goodman DS (eds): The Retinoids, vol 2. Orlando, Fla, Academic, 1984, p 177

6. Gold EJ, Mertelsmann RH, Itri LM, Gee T, Arlin Z, Kempin S, Clarkson BD, Moore MAS: Phase-I clinical trial of 13-cis-retinoic acid in myelodysplastic syndrome. Cancer Treat Rep 67:981, 1983

7. Besa EC, Granick J, Itri L, Nowell PC: Complete hematologic and cytogenetic remission in a patient with myelodysplastic syndrome treated with 13-cis-retinoic acid. Blood 62:199, 1983 (abstr)

8. Roberts AB, Sporn MB: Cellular biology and biochemistry of the retinoids, in Sporn MB, Roberts AB, Goodman DS (eds): The Retinoids, vol 2. Orlando, Fla, Academic, 1984, p 210

9. Douer D, Koeffler HP: Retinoic acid enhances colony-stimulating factor induced clonal growth of normal human myeloid progenitor cells in vitro. Exp Cell Res 138:193, 1982

10. Douer D, Koeffler P: Inhibition of the clonal growth of human myeloid leukemia cells. J Clin Invest 69:277, 1982

11. Arlin A, Berman E, Gee T, Mertlesmann R, Kempin S, Moore M, Itri L, Kurland E, Clarkson B: 13-cis-retinoic acid (RA) does not improve the true remission rate or the duration of true remission (induced with cytotoxic chemotherapy) in chronic phase chronic myelogenous leukemia (CML). Blood 62:199, 1983 (abstr)

12. Asmar S, Beranek M, Herzig G: Retinoic acid stimulates growth and affects differentiation of normal myeloid precursors. Clin Res 31:308, 1983 (abstr)

13. Januszewicz E, Zaizov R, Inbal A, Shaklai M: Selective inhibition by retinoic acid of granulocyte colony formation in the accelerated and blastic phases of chronic myeloid leukemia. Br J Haematol 58:119, 1984

14. Koeppler H, Robinson WA: 13-cis-retinoic acid enhances granulocyte-monocyte colony formation in vitro by normal human bone marrow. Blood 58:112, 1981 (abstr)

15. Nagler A, Riklis I, Tatarsky I, Fabian I: Differing responses to maturation-inducing agents in subtypes of myelodysplastic syndromes. Blood 64:170, 1984 (abstr)

16. Nillson B: Probable in vivo induction of differentiation by retinoic acid of promyelocytes in acute promyelocytic leukemia. Brit J Haematol 57:365, 1984

17. Flynn PJ, Miller WJ, Weisdorf DJ, Arthur DC, Brunning R, Branda RF: Retinoic acid treatment of acute promyelocytic leukemia: In vitro and in vivo observations. Blood 62:1211, 1983

18. Goodman DS: Vitamin A and retinoids in health and disease. N Engl J Med 310:1023, 1984

19. Kerr IG, Lippman ME, Jenkins J, Myers CE: Pharmacology of 13-cis-retinoic acid in humans. Cancer Res 42:2069, 1982

20. Feder M, Finan J, Besa E, Nowell P: A 2p;11q chromosome translocation in dysmyelopoietic preleukemia. Cancer Genet Cytogenet 15:143, 1985

21. Aye MT, Niho Y, Till JE, McCulloch EA: Studies of leukemic cell populations in culture. Blood 44:205, 1974

22. Claman HN: Corticosteroids and lymphoid cells. N Engl J Med 287:388, 1972

23. Duggan DE, Yeh KC, Matalia N, Ditzier CA, McMahon FG: Bioavailability of oral dexamethasone. Clin Pharmacol Ther 18:205, 1975

24. Brophy TR O'R, McCafferty J, Tyrer JH, Eadie MJ: Bioavailability of oral dexamethasone during high dose steroid therapy in neurological patients. Eur J Clin Pharmacol 24:103, 1983

25. Abrahm JL, Smiley R: Modification of normal human myelopoiesis by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Blood 58:1119, 1981

26. Metcalf D: Hemopoietic colonies. Recent Results Cancer Res 22:1977

27. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. Am J Clin Pathol 55:283, 1971

28. Worton RG, McCulloch EA, Till JE: Physical separation of hematopoietic stem cells from cells forming colonies in culture. J Cell Physiol 74:171, 1969

29. Seabright M: A rapid banding technique for human chromosomes. Lancet 2:971, 1971

30. Dube ID, Eaves CJ, Kalousek DK, Eaves AC: A method for obtaining high quality chromosome preparations from single hemopoietic colonies on a routine basis. Cancer Genet Cytogenet 4:157, 1981

31. Ozawa K, Sato N, Urabe A, Takaku F: Modulation of normal myelopoiesis in vitro by retinoic acid. Biochem Biophys Res Commun 123:128, 1984

32. Abrahm JL, Colucci AC: 13-cis-retinoic acid and dimethylsulfoxide inhibit monocyte differentiation of normal human colony forming units culture. Blood 58:104, 1981 (abstr)

33. Bailey-Wood R, May S, Jacobs A: The effect of retinoids on CFU-GM from normal subjects and patients with myelodysplastic syndrome. Br J Haematol 59:15, 1985

34. Findley HW, Steuber CP, Ruymann FB, Culbert S, Ragab AH: Effect of retinoic acid on the clonal growth of childhood myeloid and lymphoid leukemias: A pediatric oncology group study. Exp Hematol 12:768, 1984

35. Moore MA, Mertelsmann R, Pelus LM: Phenotypic evaluation of chronic myeloid leukemia. Blood Cells 7:217, 1981

36. Greenberg PL: The smoldering myeloid leukemic states. Clinical and biological features. Blood 61:1035, 1983

37. Hutchinson R, Long M, Appleyard J, Curnutte J, Boxer L: Severe congenital neutropenia: Spontaneous correction of the maturation arrest in long-term bone marrow cultures. Blood 64:106, 1984 (abstr)

38. Breitman TR, Selonick SE, Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL60) by retinoic acid. Proc Nat Acad Sci USA 77:2936, 1980

39. Breitman TR, Collins SJ, Keene BR: Terminal differentiation of human promyelocytic leukemia cells in primary culture in response to retinoic acid. Blood 57:1000, 1981

Trisomy 4 Identifies a Subset of Acute Nonlymphocytic Leukemias

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We present four patients with acute nonlymphocytic leukemia, one M2 and three M4 French-American-British (FAB) types and one patient with refractory anemia with excess of blasts in transformation who at diagnosis had trisomy of chromosome 4 as the primary karyotypic anomaly. This chromosome anomaly probably defines a previously undescribed subset of acute nonlymphoid leukemias. Hematologic characteristics commonly found in these patients were dysplastic features of all bone marrow lineages, suggesting that trisomy 4–associated disorders involve the early myeloid hematopoietic stem cell.

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CYTOGENETIC studies have shown that some acquired chromosomal abnormalities are characteristically associated with morphologically defined types of acute nonlymphocytic leukemia (ANLL). For example, a t(8;21) is typically associated with an M2 leukemia with granulated myeloblasts[1]; a t(15;17) is specific for both the granular and the hypogranular form of the promyelocytic M3 leukemia[2,3]; a structural anomaly of chromosome 16 involving bands p13 and q22 identifies an M4 type of acute leukemia with bone marrow eosinophilia[4]; and structural rearrangements of the long arm of chromosome 11 (11q) are nonrandomly associated with acute monocytic M5 leukemia.[5] In this paper we describe four patients with ANLL and one patient with a myelodysplastic syndrome in transformation, all associated with a trisomy of chromosome 4. The available evidence suggests that the target cell in these trisomy 4–associated disorders may be the early myeloid hematopoietic stem cell capable of differentiating along either the monocytic or the granulocytic lineage.

MATERIALS AND METHODS

Patients. Five patients with trisomy 4 were identified among a total number of 700 patients with ANLL and refractory anemia with excess blasts (RAEB) who were referred for chromosome investigation to the Centre for Human Genetics, University of Leuven, Belgium, between 1973 and 1984.

Patient N.V.C., a 17-year-old female student, was admitted for the first time in April 1974 because of persistent fever (38.5 °C), gingivitis, and otitis. Hematologic data were as follows: hemoglobin level, 10.4 g/dL; red blood cell (RBC) count, 3.2 × 10^{12}/L; WBC count, 33.7 × 10^9/L, with 52% blasts, 45% promyelocytes, 1% segmented neutrophils, and 2% lymphocytes. Bone marrow smears showed 43% blast cells. Treatment consisted of cytosine arabinoside and 6-thioguanine. The patient went into remission but relapsed after 1 month and died 9 months after diagnosis.

Patient H.B., a 64-year-old female white-collar worker, was referred in June 1984 from another center for treatment of ANLL. Hematologic data were as follows: hemoglobin level, 11.7 g/dL; and WBC count, 18.7 × 10^9/L, with 18% neutrophils, 7% monocytes, 18% lymphocytes, and 57% blasts. The platelet level was 54 × 10^9/L. A myelogram showed 6% promonocytes, 6% monocytes, 51% blast cells, 4% promyelocytes, 5% myelocytes, 2% metamyelocytes, 8% neutrophils, 1% basophils, 4% pronormoblasts, 9% normoblasts, and 4% lymphocytes. Remission was obtained after two cycles of treatment with cytosine arabinoside and daunomycin. In Sept 1984 she was readmitted with icterus and hepatomegaly because of non-A, non-B hepatitis. No signs of leukemia were found upon examination of the peripheral blood and bone marrow. At the last hematologic follow-up in May 1985 she was still in complete remission.

Patient A.Z., a 56-year-old housewife, was referred for the first time in June 1978 because of weight loss, sore throat, and fever. Physical examination revealed an enlargement of the lymph nodes in the cervical region and diffuse petechiae on the legs. There was no hepatosplenomegaly. Hematologic data were as follows: hemoglobin level, 9 g/dL; platelet count, 17 × 10^9/L; and WBC count, 248 × 10^9/L, with 76% blasts, 2% promyelocytes, 7% lymphocytes, and 15% monocytes. A myelogram showed 89% blast cells, 9% promonocytes, and 2% monocytes. The serum lysozyme level was 16 μg/mL (NV, 0 to 20) and the urine lysozyme level, 20 μg/mL (normal values [NV], 0 to 5). Therapy consisted of cytosine arabinoside, Adriamycin (Adria Laboratories, Columbus, Ohio) and 6-thioguanine. Remission was not obtained, and the patient died of bronchopneumonia 2 months after diagnosis.

Patient 4, J.M., a 15-year-old male student, was observed for the first time in Oct 1984 because of weakness. Physical examination revealed hepatosplenomegaly. Hematologic data were as follows: hemoglobin level, 7.6 g/dL; RBC count, 2.16 × 10^{12}/L; and WBC count, 4.7 × 10^9/L, with 13% blasts, 4% band neutrophils, 13% segmented neutrophils, 1% basophils, 64% lymphocytes, and 5% monocytes. The platelet count was 31 × 10^9/L. A bone marrow aspirate and bone biopsy specimen were hypercellular with a massive infiltration by blast cells. A myelogram showed 87% blasts, 2% promyelocytes, 2% myelocytes, 3% band neutrophils, 3% segmented neutrophils, 1% eosinophils, and 2% lymphocytes. Remission was obtained using a therapeutic regimen with cytosine arabinoside and daunomycin. The patient died in aplasia during consolidation therapy.

Patient P.B., a 58-year-old housewife, was first observed in July 1983. On admission she showed splenomegaly and the following hematologic data: hemoglobin level, 8 g/dL; platelet count, 199 × 10^9/L; and WBC count, 3.6 × 10^9/L, with 32% neutrophils, 2% eosinophils, 1% basophils, 53% lymphocytes, and 12% lymphocyte-like unclassifiable elements. Bone biopsy findings showed focal infiltration by small round elements with a high nuclear cytoplasmic ratio. The disease remained stationary until Oct 1984 when leukocytosis (WBC count, 15.8 × 10^9/L) and thrombocytopenia (platelet count, 85 × 10^9/L) were noted. The peripheral blood differential count consisted of 20% neutrophils, 10% monocytes, 48% lymphocytes, and 22% blast cells. Bone marrow smears showed 28% blasts. Treatment with 6-mercaptopurine was started, but it was discontinued after 1 month because severe leukopenia and septicemia developed. The last follow-up in May 1985 showed a patient in good general condition and the following hematologic data: 9.9 g/dL of

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Table 1. Clinical Data of the Patients and Morphologic, Cytochemical, and Antigenic Characteristics of Their Blast Cells at the Time of Chromosome Investigation

							Cytochemistry*										
Patient	Age/Sex	Date	FAB Type	HB (g/dL)	Platelets (× 10^9/L)	WBC (× 10^9/L)	Sample	Blasts (%)	Promyelocytes (%)	PAS	POX	SBB	ANBE	ANAE	Immunophenotype†	Therapy	Survival From Diagnosis (mo)
N.V.C.	17/F	April 1974	M2	10.4	90	33.7	BM	43	55	ND	ND	ND	ND	ND	—	ARA-C 6-TG	7, died
H.B.	64/F	June 1984	M4	11.7	54	18.7	BM	51	4	ND	40	50	ND	30	TdT-neg, BA-2-pos, OKT10-pos, OKT3-neg, SIg-neg	ARA-C Dauno	12, +
A.Z.	56/F	June 1978	M4	9	17	248	BM	89	9	0	0	10	49	ND	—	ARA-C 6-TG Adria	1.5, died
J.M.	15/M	Oct 1984	M4	7.6	31	4.7	BM	87	2	0	40	ND	30	ND	TdT-neg, OKIa1-pos, OKM5-pos	ARA-C Dauno	6, died
P.B.	58/F	Oct 1984	RAEBt	8	199	15.8	PB	22	—	0	ND	8	ND	2	TdT neg, BA-1-neg, B1-neg, J5-neg, OKT3-neg, OKIa1-pos, OKT9-pos, OKT10-pos	6-MP	22, +

Abbreviations: BM, bone marrow; PB, peripheral blood. RAEBt, refractory anemia with excess of blasts in transformation; ARA-C, cytosine arabinoside; 6-TG, 6 thioguanine; Adria, Adriamycin; Dauno, daunomycin; 6-MP, 6-mercaptopurine; ND, not determined.

*Numbers indicate positive cells among blast cells.

†Reactivity of monoclonal antibodies: TdT, lymphoid progenitor cells[8]; BA-1, pre-B cells, B cells, granulocytes[9]; BA-2, pre-B and B cells; vast majority of non-T acute lymphocytic leukemia (ALL), some cases of nonlymphoid leukemias[10]; B1, pre-B, B cells[11]; J5, common ALL antigen[12]; OKIa1, HLA-DR antigens, B cells, macrophages, activated T cells[13]; OKT3, mature T cells[14]; OKT9, transferrin receptor, proliferating cells, including stem cells and activated lymphocytes[15]; OKT10, early stem cells, activated lymphocytes[16]; OKM5, monocytes and platelets.[17]

hemoglobin, 157 × 10^9/L platelets, and 9.4 × 10^9/L WBC with 19% blast cells.

Morphologic and cytochemical studies. May-Grünwald-Giemsa (MGG)-stained slides of bone marrow and peripheral blood were reviewed retrospectively in all the cases using the criteria of the French-American-British (FAB) classification for acute leukemias and myelodysplastic syndromes.[6,7] Cytochemical studies included assays for periodic acid-Schiff stain (PAS), Sudan black B (SBB), peroxidase (POX), alpha-naphthyl-acetate-esterase (ANAE), and alpha-naphthyl-butyrate esterase (ANBE) and were performed following the usual criteria.[7]

Immunologic marker studies. Surface markers were detected by an indirect immunofluorescence technique using fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Ortho Pharmaceutical Corp, Raritan, NJ) as the second antibody. Cell surface immunoglobulins were tested by direct fluorescence with FITC-conjugated antibodies.

Monoclonal antibodies OKIa1, OKT3, OKT9, OKT10, OKM5 (Ortho Pharmaceuticals), BA-1, BA-2 (Hybritech, San Diego), B1, and J5 (Coulter Immunology, Hialeah, Fla) were obtained commercially. Reactivity is reported in Table 1. Terminal deoxynucleotidyl transferase (TdT) activity was determined by immunofluorescence using rabbit anticalf TdT serum and FITC-conjugated goat antirabbit serum (Bethesda Research Laboratories, Md).

Cytogenetic studies. Chromosome analysis was performed at diagnosis on all patients. Metaphase chromosome preparations were obtained from bone marrow and/or peripheral blood cells after 48-hour unstimulated cultures. A reverse banding with acridine orange was used. Karyotypes were arranged according to the International System for Human Cytogenetic Nomenclature.[18]

RESULTS

Morphologic, cytochemical, and immunologic studies. The age, sex, presenting symptoms, WBC count, hemoglobin level, platelet count, cytochemical properties and immunophenotype of blast cells, type of treatment, and survival from diagnosis of the five patients with trisomy 4 are listed in Table 1.

Patient N.C.V. was classified as FAB M2 on the basis of the presence of maturation of the bone marrow cells, including granulated myeloblasts, Auer's rods, and a high number of promyelocytes. Pelger-Huët–like anomaly, megaloblastic features of the red cell series, and a very few, abnormal megakaryocytes were noted.

Patient H.B. was classified at diagnosis as M4. MGG-stained slides of bone marrow showed maturation of the myeloid blasts and a few elements with single Auer's rods. Nuclear abnormalities with bilobated or clover nuclei were prominent in the blast elements. Bone marrow smears showed 30% of the blast cells positive for ANAE. The more differentiated granulocytic forms showed a Pelger-Huët–like anomaly as well as hypersegmentation. A few eosinobasophilic elements were also observed. The red cell series was megaloblastic, and several binucleated forms were present. Megakaryocytes were abnormal with monolobulated nuclei and cytoplasmic basophilia. Blast cells in this case reacted with the BA-2 monoclonal antibody. All other lymphoid markers, however, were negative, including TdT, T3 antigen, and surface immunoglobulins.

In patient A.Z., bone marrow was classified as M4 and showed abnormal maturation of the myeloid series with hypogranulation. Pelger-Huët–like anomaly was present. Several blast cells showed scanty cytoplasm and sometimes folded nuclei. Most of these elements (49%) showed ANBE activity partially inhibited by NaF. Megaloblastosis of red cell series, hypolobulation of megakaryocytes, and a few eosinobasophils were associated morphologic anomalies.

Patient J.M. showed a typical FAB M4. ANBE activity was present in 30% of the immature elements. Morphologic anomalies, ie, megaloblastosis of the red cell series, dysmegakaryopoiesis, Pelger-Huët–like anomalies, and eosinobasophilic elements were consistently present in this case.

In patient P.B., the diagnosis was RAEBt in Oct 1984 when cytogenetic studies were performed. Morphologically, two types of blast cells were identified: small blastic elements with scanty cytoplasm, diffuse nuclear chromatin, and one or sometimes two nucleoli; and blasts with folded monocytoid nuclei. There was abnormal maturation of the myeloid series with a Pelger-Huët–like anomaly. Dysmegakaryopoiesis was present with both monolobulated and apparently polynucleated forms. The red cell series was megaloblastic. Iron staining did not show ringed sideroblasts. Plasma cells and eosinobasophils were noted. The myeloid nature of the disorder in this patient was confirmed by positivity of the SBB stain in more than 5% of the blast cells. Moreover, immunophenotype studies revealed a negativity of the antibodies reacting with lymphoid antigens, ie, TdT, BA-1, B1, J5, and OKT3.

Cytogenetic studies. The karyotypic investigations of the five patients at diagnosis and during the course of the disease are summarized in Table 2. Figure 1 shows the trisomy 4 in a partial karyotype of the five patients. In patients 1, 2, and 4, the predominant clone with trisomy 4 was associated with normal karyotypes at the time of diagnosis. In patient 1, no mitoses were obtained at the time of a possible hematologic remission, but trisomy 4 was again present in 80% of the bone marrow metaphases during the terminal phase of the disease. Patient 3 showed trisomy 4 as sole anomaly in 23 out of 25 metaphases. In this patient, however, also one subclone with an additional trisomy 7 was identified. All five metaphases obtained from the peripheral blood of patient 5 showed a 47, XX, +4 karyotype.

DISCUSSION

To the best of our knowledge, trisomy 4 as the primary karyotypic anomaly has never been described in human hematologic malignancies. A few cases of ANLL, namely, one M2 and five M5 FAB types have been described in which trisomy 4 was part of a complex abnormal karyotype with both numerical and structural anomalies of other chromosomes.[19-22]

The present study documents for the first time the existence of a unique hematologic disorder associated with a trisomy of chromosome 4. This hematologic disorder is nonlymphoid in nature as demonstrated on the basis of morphology, cytochemistry, and monoclonal antibody reactivity of the blast cells. Furthermore, the fact that the acute leukemias in this study showed either granulocytic or monocytic differentiation, ie, M2 and M4 FAB types, indicates

Table 2. Cytogenetic Investigations in Five Patients With ANLL and Trisomy 4

Patient	Status of Disease	Source of Cells	Culture (h)	Abnormal Karyotypes	Number of Normal Karyotypes
N.C.V.	diagnosis	BM	48	47, XX +4 (16/24)	8
		PB	48	47, XX, +4 (3/17)	14
	remission	BM	48	no mitoses	
	relapse	BM	48	47, XX, +4 (20/25)	5
H.B.	diagnosis	PB	48	47, XX, +4 (14/15)	1
	remission	BM	48	47, XX, +4 (1/17)	16
A.Z.	diagnosis	BM	48	47, XX, +4 (23/25)	—
				48, XX, +4, +7 (2/25)	—
J.M.	diagnosis	BM	48	47, XY, +4 (8/13)	5
P.B.	diagnosis	PB	48	47, XX, +4 (5/5)	—

Figures in parentheses are the number of karyotypes over the total number of metaphases studied.

that at least the common granulomonocytic progenitor cell was involved. Yet, from this study two lines of evidence suggest that an early myeloid precursor, possibly the myeloid hematopoietic stem cell, multipotent for erythrocytes, platelets, granulocytes, and monocytes, was the primary site of lesion for the trisomy 4–associated disorders. First, in all our cases of ANLL, namely, one M2 and three M4, the erythrocytic and megakaryocytic cells showed a marked dysplasia consistent with the involvement of both these series in the neoplastic process. Fialkow and colleagues,[23] using G6PD isoenzyme studies, have demonstrated that ANLLs with such morphologic features arose from a pluripotent myeloid cell. Furthermore, the last patient of our series showed a myelodysplastic syndrome that is a disorder of a pluripotent stem cell according to both morphologic and enzymatic studies.[24,25] Trisomy 4 thus probably arises in a myeloid totipotent stem cell that maintains the capacity of differentiating along the granulocytic and monocytic pathways. Dysplasia of the three bone marrow cell lines is often observed in cases of ANLL or myelodysplastic syndrome arising in patients who have a history of occupational exposure to toxics or prior cytotoxic therapy.[26] Our five patients with trisomy 4, however, were not professionally exposed to toxics. Moreover, in all our cases, cytogenetic studies were performed before any chemotherapy.

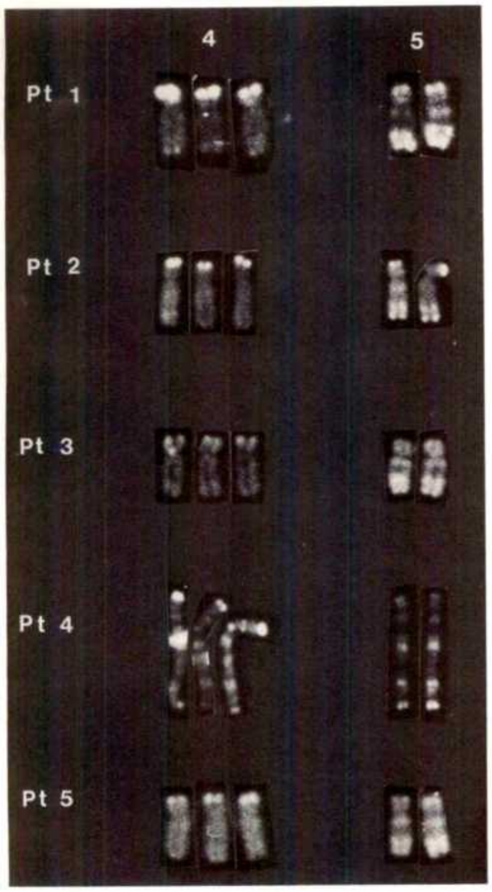

Fig 1. Partial karyotype of chromosome 4 and 5 (group B) from the five patients with trisomy 4. R-banding with acridine orange.

Finally, on the basis of our experience and previously reported large series of ANLL patients,[1,27] trisomy 4 associated disorders appear to be uncommon. In our cases, however, the trisomy 4 was found after 48 hours of culture, which is rarely used in cytogenetic laboratories. This culture method might be especially suitable in determining the trisomy 4 anomaly.

The significance of a trisomy of chromosome 4 for the biology of the malignant clone is unclear at present. Trisomy may be associated with quantitative changes in the production of specific gene products. A gene dosage effect has been described in some disorders associated with trisomy 8 and increased levels of the enzyme glutathione reductase, the gene of which is located on the short arm of chromosome 8.[28,29] A similar example was recently given by Koprowski et al[30] who demonstrated that the epidermal growth factor receptor, coded for by chromosome 7, was expressed by only melanoma cells with a trisomy 7. Therefore, one may speculate that trisomy 4 is involved in the abnormal proliferation of the neoplastic clone through an increased production of one or more factor(s) that are controlled by genes on this chromosome. Two genes involved in the proliferation and self-perpetuation of malignant cells[31] have been mapped on chromosome 4, one coding for the T cell growth factor,[32] the other for the epidermal growth factor.[33] It remains to be tested whether trisomy 4 confers a proliferating advantage to the malignant cells by amplification of one or more of these growth factor(s).

REFERENCES

1. Fourth International Workshop on Chromosomes in Leukemia: A prospective study of acute nonlymphocytic leukemia. Cancer Genet Cytogenet 11:249, 1984

2. Van den Berghe H, Louwagie A, Broeckaert-Van Orshoven A, David G, Verwilghen R, Michaux JL, Ferrant A, Sokal G: Chromosome abnormalities in acute promyelocytic leukemia (APL). Cancer 43:558, 1979

3. Golomb HM, Rowley JD, Vardiman JW, Testa JR, Butler A: "Microgranular" acute promyelocytic leukemia: A distinct clinical, ultrastructural and cytogenetic entity. Blood 55:253, 1980

4. Le Beau MM, Larson RA, Bitter MA, Vardiman JW, Golomb HM, Rowley JD: Association of an inversion of chromosome 16 with abnormal marrow eosinophils in acute myelomonocytic leukemia: A unique cytogenetic-clinicopathological association. N Engl J Med 309:630, 1983

5. Berger R, Bernheim A, Weh HJ, Daniel MT, Flandrin G: Cytogenetic studies on acute monocytic leukemia. Leuk Res 4:119, 1980

6. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukaemias. French-American-British (FAB) cooperative group. Br J Haematol 33:451, 1976

7. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C (FAB cooperative group). Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

8. Bollum FJ: Terminal deoxynucleotydyl transferase as a hematopoietic cell marker. Blood 54:1203, 1979

9. Abramson C, Kersey J, Le Bien T: A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. J Immunol 126:83, 1981

10. Le Bien TW, Kersey JH: Utilization of monoclonal antibodies in the study of cell surface antigens on human B lymphocytes, in Haynes BF, Eisenbarth GS (eds): Monoclonal Antibodies: Probes for the Study of Autoimmunity and Immunodeficiency. Orlando, Fla, Academic, 115, 1983

11. Stashenko P, Nadler LM, Hardy R, Schlossman SF: Characterization of a human B lymphocyte specific antigen. J Immunol 125:1678, 1980

12. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF: A monoclonal antibody to human acute lymphoblastic leukemia antigen. Nature 283:583, 1980

13. Reinherz EL, King PC, Pesando JM, Ritz J, Goldstein G, Schlossman JF. In determinants on human T cell subsets defined by monoclonal antibody: Activation stimuli required for expression. J Exp Dev 150:1472, 1979

14. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF: Discrete stages of human intrathymic differentiation: Analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA 77:1588, 1980

15. Terhorst C, Van Agthoven A, LeClair K, Snow P, Reinherz E, Schlossman S: Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. Cell 23:771, 1981

16. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G: Distribution of T lymphocyte subsets in the human bone marrow and thymus: An analysis with monoclonal antibodies. J Immunol 126:1608, 1981

17. Shen HH, Talle MA, Goldstein G, Chess L: Functional subsets of human monocytes defined by monoclonal antibodies: A distinct subset of monocytes contains the cells capable of inducing the autologous mixed lymphocyte culture. J Immunol 130:698, 1983

18. An international system for human cytogenetic nomenclature (1978): Report of the Standing Committee on Human Cytogenetic Nomenclature. Cytogenet Cell Genet 21:309, 1978

19. Löwenberg B, Hagemeijer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641, 1982

20. Kaneko Y, Rowley JD, Maurer HS, Variakojis D, Moohr JW: Chromosome pattern in childhood acute nonlymphocytic leukemia (ANLL). Blood 60:389, 1982

21. Li YS, Khalid G, Hayhoe FGJ: Correlation between chromosomal pattern, cytological subtypes, response to therapy, and survival in acute myeloid leukemia. Scand J Haematol 30:265, 1983

22. Dewald G, Morrison-Dehop SJ, Schuchard KA, Spurbeck JL, Pierre R: A possible specific chromosome marker for monocytic leukemia: Three more patients with t(9;11)(p22;q24) and another with t(11;17)(q24;q21), each with acute monoblastic leukemia. Cancer Genet Cytogenet 8:203, 1983

23. Fialkow PS, Singer JW, Adamson JW, Vaidyva K, Dow LW, Ochs J, Moohr JW: Acute non lymphocytic leukemia: Heterogeneity of stem cell origin. Blood 57, 1068, 1981

24. Prchal JT, Trockmorton DW, Carrol AJ, Fuson EW, Gams RA, Prchal JF: A common progenitor for myeloid and lymphoid cells. Nature 274:590, 1978

25. Heimpel H: Conventional morphological examination of blood and bone marrow in the diagnosis of preleukemic syndromes, in F. Schmalz, KP Hellriegel (eds): Preleukemie. Berlin, Springer-Verlag, 1979, p 4

26. Michels SD, McKenna RW, Arthur DC, Brunning RD: Therapy-related acute myeloid leukemia and myelodysplastic syndrome: A clinical and morphological study of 65 cases. Blood 65:1364, 1985

27. Yunis JJ, Bloomfield CD, Ensrud K: All patients with acute nonlymphocytic leukemia may have a chromosomal defect. N Engl J Med 305:135, 1981

28. Jensen PKA, Junien C, Despoisse S, Bernsen A, Thelle T, Friedrich V, De La Despoisse A: Inverted tandem duplication of the short arm of chromosome 8: A non-random de novo structural aberration in man. Localization of the gene for glutathione reductase in subband 8p21.1. Ann Genet (Paris) 25:207, 1982

29. de la Chapelle A, Vuopio P, Icén A: Trisomy 8 in the bone marrow associated with high red cell glutathione reductase activity. Blood 47:815, 1976

30. Koprowski H, Herlyn M, Balaban G, Parmiter A, Ross A, Nowell P: Expression of the receptor of epidermal growth factor correlates with increased dosage of chromosome 7 in malignant melanoma. Somatic Cell Mol Genet 11:297, 1985

31. Sporn MB, Roberts AB: Autocrine growth factors and cancer. Nature 313:745, 1985

32. Seigel LJ, Harper ME, Wong-Staal F, Gallo R, Nash WG, O'Brien SJ: Gene for T-cell growth factor: Location on human chromosome 4q and feline chromosome B1. Science 223:175, 1984

33. Zabel BU, Eddy RL, Lalley PA, Scott J, Bell GI, Shows TB: Chromosomal locations of the human and mouse genes for precursors of epidermal growth factor and the β subunit of nerve growth factor. Proc Natl Acad Sci USA 82:469, 1985

Proteoglycans in Human Long-Term Bone Marrow Cultures: Biochemical and Ultrastructural Analyses

By Thomas N. Wight, Michael G. Kinsella, Armand Keating, and Jack W. Singer

Proteoglycans within the extracellular matrix of human bone marrow have been implicated in the process of hematopoiesis, but little is known about the structure and composition of these macromolecules in this tissue. Hematopoietically active human long-term bone marrow cultures were incubated with medium containing ^{35}S-sulfate and ^{3}H-glucosamine as labeling precursors. Proteoglycans present in the medium and cell layer were extracted with 4 mol/L guanidine HCl and purified by diethylaminoethyl (DEAE)-Sephacel ion exchange and molecular sieve chromatography. Both culture compartments contain a large chondroitin sulfate proteoglycan (MI, CI) that eluted in the void volume of a Sepharose CL-4B column and contained glycosaminoglycan chains of molecular weight (mol wt) ~38,000. A second population of sulfate-labeled material was identified as a broad heterogenous peak (MII, CII) that was included on Sepharose CL-4B at K_{av} = 0.31. This material when chromatographed on Sepharose CL-6B could be further separated into a void peak (MIIa, CIIa) and an included peak eluting at K_{av} = 0.39 (MIIb, CIIb). The void peaks (MIIa, CIIa) were susceptible to chondroitinase ABC digestion (99%) but slightly less susceptible to chondroitinase AC digestion (90%). Papain digestion of these peaks revealed them to be proteoglycans with glycosaminoglycan chains of mol wt ~38,000. The included peaks on Sepharose CL-6B (MIIb, CIIb) from both medium and cell layer compartments resisted digestion with papain, indicating the presence of glycosaminoglycan chains of mol wt ~38,000 either free or attached to a small peptide. Although this material was susceptible to chondroitinase ABC (98%), it was considerably less susceptible to chondrotinase AC (~60%), indicating that it contained dermatan sulfate. A small amount of heparan sulfate proteoglycan was also identified but constituted only ~10% of the total sulfated proteoglycan extracted from these cultures. Additionally, approximately 40% of the incorporated ^{3}H-activity radioactivity was present as hyaluronic acid. Electron microscopy revealed a layer of adherent cells covered by a mat containing ruthenium red-positive granules that were connected by thin filaments. The extracellular matrix layer above the adherent cells contained a mixture of hematopoietic cells. Chondroitinase ABC treatment of the cultures completely removed the ruthenium red-positive granules overlying the cells and resulted in a loss of ~70% of the ^{35}S-sulfate-labeled material from the cell layer. This culture system is offered as a model to investigate the role of proteoglycans in hematopoiesis.

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THE MICROENVIRONMENT of hematopoietic organs is thought to play a fundamental role in promoting and sustaining hematopoiesis.[1,2] Recently, long-term bone marrow cultures have been developed and used to demonstrate that, in some systems, a class or family of adherent cells, collectively termed stromal cells, are necessary to support the proliferation and differentiation of pluripotent hematopoietic stem cells in vitro.[3-5] The mechanism by which these adherent cells influence hematopoiesis is not known, but the morphologic similarities between the microenvironmental cells present in vivo and those in the stromal cell layer of long-term marrow cultures[6] make this culture system a valuable model to study those factors of the microenvironment that may regulate hematopoiesis.

One component of the hematopoietic microenvironment that has been implicated in hematopoiesis is a group of complex carbohydrates termed proteoglycans. These macromolecules consist of a protein core to which glycosaminoglycans and oligosaccharides are covalently attached.[7] A number of different families of proteoglycans exist within a variety of tissues and are usually defined by their core protein and glycosaminoglycan composition. Such families are found associated with plasma membranes and within the interstitial matrix. These macromolecules have been shown not only to affect the structural integrity of a number of different tissues but also to influence many cell processes such as proliferation and migration, which are fundamental to morphogenesis and differentiation.[8] Early clinical and experimental studies of starvation-induced defective hematopoiesis revealed excessive accumulation of proteoglycans within hematopoietic organs.[9,10] Subsequent studies using animal models of anemia (Sl/Sl^{d} mice) demonstrated elevated levels of chondroitin sulfate and hyaluronic acid within the spleens of affected animals.[11,12] These early studies led to the hypothesis that accumulation of proteoglycans within hematopoietic organs suppressed hematopoiesis. Additional support for this hypothesis was obtained when Pleomacher et al[13] demonstrated inhibition of erythrocytic differentiation in vitro by pretreating cultures with elevated levels of glycosaminoglycans (hyaluronic acid, chondroitin sulfate, dermatan sulfate, and heparan sulfate). The finding that chondroitin 4-sulfate is present on the surface of mature hematopoietic cells but missing on immature blast cells as well as peripheral blood leukocytes from leukemic patients[14] emphasizes the potential importance of cell surface glycosaminoglycan (proteoglycan) in regulating hematopoiesis. Additional studies by del Rosso et al[15] illustrate that the

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attachment of early hematopoietic cells to an adherent cell layer in vitro can be blocked by removing cell surface glycosaminoglycans from the hematopoietic cells and/or adherent cells by specific glycosidases. Thus, proteoglycans appear to regulate stromal cell–hematopoietic cell interaction.

To date, the majority of studies implicating proteoglycans or their constituent glycosaminoglycans in hematopoiesis have been indirect (ie, examining glycosaminoglycan content in systems exhibiting defective hematopoiesis). Recently, a more direct approach has been taken by Dexter and his colleagues in experiments in which proteoglycan synthesis in long-term mouse bone marrow cultures has been perturbed by treatment with β-D-xyloside. Modification of proteoglycan synthesis in these cultures was associated with an increased production of stem cells.[16] Since xylosides increase free-chain glycosaminoglycan synthesis but decrease overall proteoglycan synthesis,[17-19] the nature of the proteoglycan effect responsible for the elevated level of hematopoiesis in this culture system is not yet clear.

Little is known about the types of proteoglycans present in hematopoietic systems since previous studies have analyzed only glycosaminoglycans of hematopoietic tissues and have failed to consider the nature of the entire proteoglycan molecule in which the glycosaminoglycans are found. Since glycosaminoglycans are synthesized within the tissue, not as free chains but as proteoglycan monomer, knowledge of the molecular structure of proteoglycans within hematopoietic tissue would appear essential if their role in hematopoiesis is to be clarified. Recent studies by Thompson and Spooner[20] demonstrated that inhibition of branching morphogenesis in mouse salivary glands was correlated with decreased proteoglycan synthesis rather than with increased free-chain glycosaminoglycan synthesis. These studies highlight the importance of native proteoglycan structure in events associated with morphogenesis.

The objective of this study was to characterize proteoglycans synthesized and secreted by hematopoietically active long-term human bone marrow cultures and to determine whether these macromolecules are localized to specific extracellular domains within these cultures. These studies will serve as a basis for future studies designed to test the hypothesis that proteoglycans within hematopoietic organs regulate hematopoiesis.

MATERIALS AND METHODS

Materials

Guanidine HCl, cesium chloride, Tris-HCl, and papain were all purchased from Sigma Chemical Co, St Louis; 6-aminohexanoic acid and benzamidine HCl from Eastman Kodak Co, Rochester, NY; chondroitinase AC-II (*Arthrobacter aurescens*), chondroitinase ABC (*Proteus vulgaris*) from Seikagaku, Kogyo, Japan, through Miles Laboratories, Inc, Naperville, Ill; *Streptomyces* hyaluronidase from CalBiochem-Behring Corp, San Diego; Sephadex, Sepharose, and diethylaminoethyl (DEAE)-Sephacel were from Pharmacia Fine Chemicals, Inc, Piscataway, NJ; $Na_2{}^{35}SO_4$ (10 mCi/mL:379 mCi/mol/L), D-[6-^{3}H]-glucosamine (1.0 mCi/mL:31 Ci/mol/L) were from New England Nuclear, Boston; AquaMix from WestChem Products, San Diego; urea from MCB, Cincinnati; McCoy's complete medium and horse serum from GIBCO, Grand Island, NY; fetal bovine serum and hydrocortisone from Reheis Chemical Co, Kankakee, Ill; Falcon tissue culture dishes from Falcon Labware, Oxnard, Calif.

Cell Culture

Marrow aspirates, drawn into 10 U/mL of preservative-free heparin were obtained from five normal marrow transplant donors. Marrow buffy coat cells were prepared, and long-term cultures were generated from the marrow of each donor using a modification of the Gartner and Kaplan method.[5] Briefly, 20 × 10^6 marrow buffy coat cells were seeded into 25-cm^2 tissue culture flasks containing McCoy's modified complete medium with 12.5% fetal bovine serum, 12.5% horse serum, and 1.0 µmol/L hydrocortisone at 2 × 10^6 cells/mL. The cultures were incubated at 37 °C in 5% CO_2 for 1 week at 37 °C and then transferred to 33 °C. Half the medium was removed weekly after gentle agitation of the flasks and replaced with fresh medium. The nonadherent cells removed with the medium were assayed for committed hematopoietic progenitors.[21] Long-term marrow cultures were studied for proteoglycan biosynthesis at 4 to 5 weeks when all cultures had confluent stromal cell layers and were producing committed hemotopoietic progenitor cells at optimal levels.

Radioisotopic Labeling of Cultures and Extraction of Proteoglycans

Cell cultures were labeled by the addition of fresh medium containing $Na_2{}^{35}SO_4$ (50 µCi/mL) and ^{3}H-glucosamine (20 µCi/mL) for times ranging from eight to 72 hours. After labeling, the medium fraction was removed and frozen at −70 °C after the addition of solid guanidine-HCl (GuHCl) (.53 g/mL) to bring the solution to approximately 4 mol/L. The cell layer of each culture was extracted with 4 mol/L GuHCl (dissociative solvent) containing 0.1 mmol/L 6-aminohexanoic acid, 5 mmol/L benzamidine HCl, 50 mol/L sodium EDTA, and 50 mmol/L sodium acetate, pH 5.8. This results in removal of 95% of the macromolecular ^{35}S activity from the cell layer, with 5% of the total ^{35}S activity associated with an insoluble residue (as determined by NaOH extraction of the insoluble cell residue). The cell layers were disrupted by scraping with a rubber policeman and immediately frozen at −70 °C until further analysis.[22]

Purification and Characterization of Proteoglycans

DEAE-Sephacel ion-exchange chromatography. Aliquots (5 mL) of 4 mol/L GuHCl extracts of the medium and cell layer were eluted on a Sephadex G-50 column (0.9 × 45 cm) equilibrated with 8 mol/L urea, 0.10 mol/L NaCl, 0.05 mol/L Tris-HCl, pH 7.5, 2 mol/L EDTA, and 0.5% Triton X-100.[23] Portions of the excluded peaks were counted for radioactivity to determine recoveries and distributions of labeled macromolecules in the medium and cell layer. The remainder of the excluded fractions were pooled (18.0 mL) and applied to a 3.0-mL column of DEAE-Sephacel equilibrated with the same solvent used for elution on Sephadex G-50. After the sample was applied and washed with 10 mL of solvent, a continuous 0.1 to 0.7 mol/L NaCl gradient was applied in the same solvent using a total of 60 mL. Fractions of 0.9 mL were collected at a flow rate of approximately 3.6 mL/h, and aliquots were counted for radioactivity. For both medium and cell layer, a major ^{3}H-labeled peak (A) and two ^{35}S-labeled peaks (B and C) were identified.

Enzymatic and chemical treatment An aliquot of each of the labeled peaks (A, B, and C) was dialyzed against distilled water, lyophilized, and subjected to one of the following treatments:

Streptomyces hyaluronidase (50 turbidity reducing units [TRU]/mL in 0.5 mol/L sodium acetate buffer, pH 5.2, for four hours at 22 °C) to identify hyaluronic acid[24]; nitrous acid degradation at a low pH to identify heparan sulfate[25]; and chondroitinase ABC or chondroitinase AC (25 U/mL in enriched Tris buffer, pH 8.0, for three hours at 37 °C) to identify chondroitin sulfate and/or dermatan sulfate.[26] The extent of digestion was assessed by separating labeled digestion products from undigested material using Sephadex G-50 columns (0.7 × 30 cm) with 0.1 mol/L ammonium acetate in 20% ethanol as the eluting buffer.

Isopycnic CsCl centrifugation. A 300-μL aliquot of peak C was added to a 4 mol/L GuHCl solution (~4.0 mL), and the mixture was brought to an initial starting density of 1.46 g/mL by adding solid CsCl (0.5 g/g).[22] Dissociative density gradients were formed by centrifuging at 37,000 rpm at 10 °C for 65 hours in an SW 50.1 rotor (Beckman Instruments, Inc, Fullerton, Calif). Gradients were divided into four equal fractions (D1, D2, D3, D4) using a Beckman tube slicer, and an aliquot from each fraction was counted for radioactivity.

Molecular sieve chromatography. For both medium and cell layer, portions of the major ^{35}S-labeled peak (peak C, see Fig 2) were concentrated first by diluting pooled fractions (~20 ml) with an equal volume of 8 mol/L urea buffer that did not contain NaCl to reduce NaCl concentration. This mixture was then reapplied to a DEAE-Sephacel column (bed volume, 0.6 mL). After sample application, the column was extracted with a small volume (0.6 to 0.8 mL) of 4 mol/L GuHCl buffer containing 0.5% Triton X-100.[27] Recovery for the concentration step was between 80% and 90%. The concentrated sample was applied to a preparative Sepharose CL-4B column (0.9 cm × 58 cm) and eluted with 4 mol/L GuHCl, 0.1 mol/L Tris-HCl, 0.1 mol/L Na$_2$SO$_4$, and 2.5 mmol/L EDTA, pH 7.0. Individual peaks were identified by counting small aliquots of each fraction, and fractions from each peak were then pooled and concentrated by contact desiccation. Proteoglycan carrier (100 μL of 10 mg/mL) prepared from rat chondrosarcoma[27] was added to each concentrated peak obtained from the Sepharose CL-4B column and rechromatographed on a preparative Sepharose CL-6B column (0.9 cm × 58 cm) eluted with 4 mol/L GuHCl as previously described. Peak fractions were identified by scintillation counting, pooled and concentrated by contact desiccation. The concentrated peaks were desalted on Sephadex G-50 (bed volume, 10 mL) and proteoglycans present in each peak were precipitated with 3 vol of ethanol containing 1.3% potassium acetate. Each precipitated peak was digested with either chondroitinase ABC or AC as previously described and digestion products separated by chromatography on Sephadex CL-6B (0.7 cm × 110 cm) eluted with 4 mol/L GuHCl, pH 7.0.

Glycosaminoglycans in each proteoglycan population were released from the core proteins by papain digestion (30 μg/mL) in 0.1 mol/L sodium acetate, 5 mmol/L EDTA, 5 mol/L cysteine, pH 7.0, for four hours at 65 °C.[28] Aliquots of the released glycosaminoglycans were chromatographed on a Sepharose CL-6B column (0.7 × 100 cm) eluted with 0.5 mol/L NaAc, 2.5 mmol/L EDTA, pH 7.0. Fractions of 0.75 mL were collected and taken for measurement of radioactivity. The excluded (V$_0$) and total (V$_t$) volumes of the column were determined by using ^{3}H-labeled DNA and free ^{35}S-sulfate, respectively. The average molecular weight of the released glycosaminoglycans was estimated by comparing the elution position (partition coefficient = K$_{av}$) of the sample fractions against known chondroitin sulfate standards as described by Wasteson.[29]

Transmission and scanning electron microscopy. Culture medium was removed, and representative cultures were rinsed briefly with phosphate-buffered saline (PBS) and fixed *in situ* with one-half strength Karnovsky's fixative buffered with 0.1 mol/L cacodylate, pH 7.3, containing 0.2% ruthenium red (Pelco, Inc, Tustin, Calif) for one hour at room temperature.[30,31] Primary fixation was followed by a rinse with 0.1 mol/L cacodylate and 7.5% sucrose buffer containing 0.1% ruthenium red for 30 minutes at room temperature and postfixation, in 0.1 mol/L cacodylate-buffered 1% osmium tetroxide containing 0.05% ruthenium red for 30 minutes at room temperature. Following fixation, the cultures were dehydrated in graded ethanols and either embedded in epoxy resins for transmission electron microscopy observations or critical point dried and coated for scanning electron microscopy observation.[32] Thin longitudinal and cross sections of the cultures were cut with diamond knives on an Ultratome III (LKB, Gaithersburg, Md), double stained with uranyl acetate and lead citrate, and examined in either an AEI-801 or JEOL 100B electron microscope. For scanning electron microscopy, specimens were observed using a JSM 35C (JEOL, Boston, Mass) scanning electron microscope.

The specificity of the staining procedure for identifying proteoglycans was examined by incubating representative cultures with chondroitinase ABC (0.5 U/mL in 0.2 mol/L enriched Tris buffer in the presence of protease inhibitors[33]) for 30 minutes at 37 °C. The cultures were rinsed with PBS, fixed, and processed as previously described. Controls were incubated with chondroitinase ABC buffer without enzyme.

To determine the percentage of labeled proteoglycans removed from the cell layer following chondroitinase ABC treatment, cultures were prelabeled with ^{35}S-sulfate for 72 hours as previously described. Following labeling, the culture media were carefully decanted, and the cell layers were incubated in the presence or absence of chondroitinase ABC (0.25 u/mL) in enriched Tris-HCl buffer, pH 7.4, for 30 minutes at 37 °C. The amount of ^{35}S-sulfate macromolecular radioactivity present in the incubation medium and in 4 mol/L guanidine HCl extracts of the cell layer was determined after Sephadex G-25 (PDIO) chromatography by scintillation counting.

RESULTS

Accumulation of ^{35}S-Sulfate–Labeled Macromolecules

The amount of ^{35}S-sulfate radioactivity incorporated into macromoluecules released into the medium and present in the 4 mol/L GuHCl extracts of the cell layer of human bone marrow cultures are shown in Fig 1. The accumulation of ^{35}S-sulfate–labeled macromolecules in the medium was nearly linear throughout a 72-hour labeling period. On the other hand, the cell layer accumulated ^{35}S-sulfate radioactivity linearly for the first 24 hours and then reached a plateau, staying fairly constant up to 72 hours. By 72 hours, approximately 70% of the macromolecular ^{35}S-sulfate activity was present in the medium, with the remainder deposited in the cell layer. The amount of DNA in these cultures did not increase during the 72-hour labeling period (data not shown).

Proteoglycan Isolation and Characterization

Portions of 4 mol/L GuHCl extracts of the cell layers and media were desalted and equilibrated in an 8 mol/L urea buffer by Sephadex G-50 chromatography.[23] The excluded volume fractions were applied to DEAE-Sephacel in the same solvent and eluted with a continuous NaCl gradient. The profiles of radioactivity eluting from the columns were similar for the medium and cell layer fractions (Fig 2). Approximately 40% to 45% of the applied ^{3}H radioactivity in

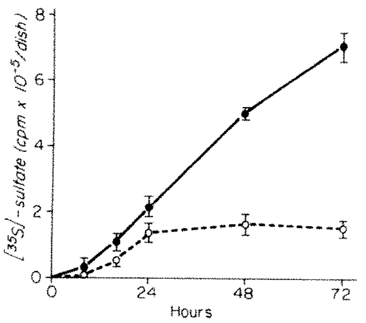

Fig 1. Accumulation of ^{35}S-sulfate–labeled macromolecules in the culture medium (solid circles) and cell layer (open circles) for a 72-hour period in 3- to 4-week bone marrow cultures.

the medium and cell layer did not bind to the ion-exchange column, eluting ahead of the salt gradient in the wash as a glycoprotein fraction. Less than 3% macromolecular ^{35}S-sulfate activity eluted in this fraction. In both medium and cell layer samples a large peak (A) containing only ^{3}H activity eluted early (~0.26 mol/L NaCl) and was followed by two closely spaced peaks containing ^{3}H and ^{35}S-sulfate activity (peaks B and C). Peak A was the predominant ^{3}H-labeled peak for both medium and cell layer (~38% of the total counts). Peak C was the predominant ^{35}S-sulfate–labeled peak in both the medium and cell layer and contained approximately 89% to 94% and 83% to 91% of the total ^{35}S-sulfate activity, respectively. The remainder of the ^{35}S-sulfate activity was present in peak B for both culture compartments.

Peaks A, B, and C from the DEAE columns were further pooled, dialyzed, and subjected to *Streptomyces* hyaluronidase digestion[24] to identify hyaluronic acid, chondroitinase ABC or AC II,[26] chondroitin sulfate and dermatan sulfate or nitrous acid,[25] and the presence of heparan sulfate. Digestion products were separated on Sephadex G-50 columns (Fig 3). Peak A from both the medium and cell layer was >95%

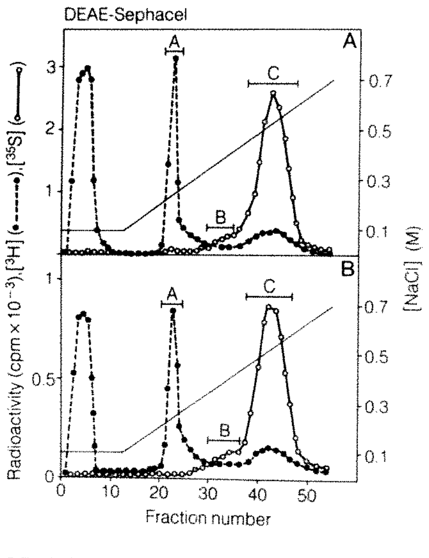

Fig 2. DEAE-Sephacel ion-exchange chromatographic profiles of ^{35}S-sulfate– and ^{3}H-glucosamine–labeled macromolecules present in 4 mol/L GuHCl extracts of medium (A) and cell layer (B). The 4 mol/L GuHCl extracts were desalted on Sephadex G-50 columns into 8 mol/L urea in Tris-HCl, pH 7.5, with 0.5% Triton X-100 and 0.1 mol/L NaCl and applied to DEAE in the same buffer. The radioactivity was eluted during a continuous (0.1 to 0.7 mol/L) NaCl gradient. Peaks B and C were pooled as indicated for further study.

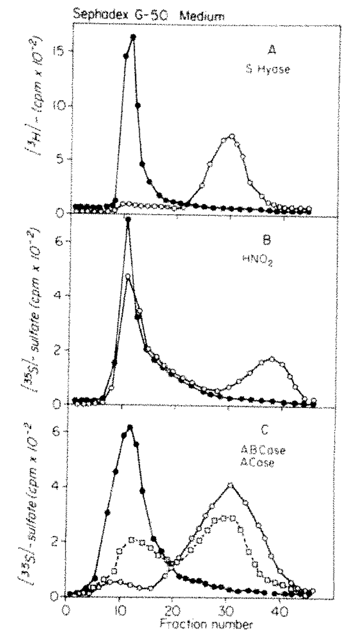

Fig 3. Sephadex G-50 profiles of pooled DEAE peaks with and without enzymatic or nitrous acid degradation. (A) Peak A, treated with *Streptomyces* hyaluronidase (open circles) and control aliquot, similarly treated in buffer without enzyme (closed circles). (B) Peak B, treated with nitrous acid (open circles) and control aliquot (closed circles). (C) Peak C, treated with either chondroitinase ABC (open circles), chondroitinase AC (squares), or buffer alone (closed circles).

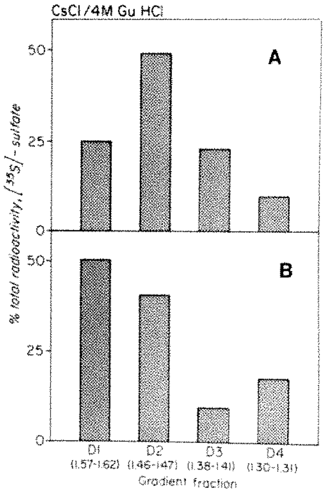

Fig 4. Distribution of ^{35}S-sulfate–labeled peak C obtained from DEAE-Sephacel ion-exchange chromatography following separation in a dissociative CsCl density gradient centrifugation. Each gradient was separated into four equal fractions from top (D4) to bottom (D1). (A), medium compartment; (B), cell layer compartment. The specific gravity of each fraction is given in parentheses.

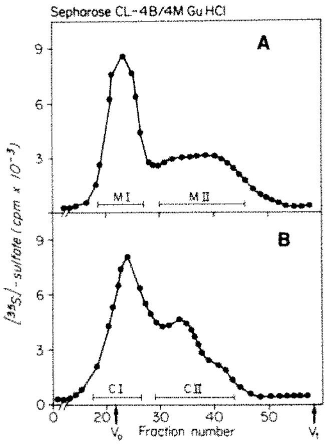

Fig 5. Preparative Sepharose CL-4B elution profiles of peak C from the medium (A) and cell layer (B) run under dissociative conditions (4 mol/L GuHCl). Bars represent those fractions that were pooled for further analysis.

susceptible to *Streptomyces* hyaluronidase, indicating that this peak contained hyaluronic acid (Fig 3A). Peak B from the cell layer and medium was partially susceptible to nitrous acid (30% to 40%) (Fig 3B), with the remainder of activity sensitive to chondroitinase ABC (not shown). The major sulfate peak from both the medium and cell layer (peak C) was almost totally susceptible to chondroitinase ABC (~86% to 93%) and partially sensitive to chondroitinase AC II (~47%) (Fig 3), indicating the presence of both chondroitin sulfate and dermatan sulfate. Aliquots of the major sulfate-labeled peak (C) from the medium and cell layer were centrifuged in a 4 mol/L GuHCl CsCl density gradient (Fig 4). The bulk of the radioactivity (>75%) was recovered in the high-buoyant density fractions of D1 (ρ = 1.550) and D2 (ρ = 1.473), indicating that proteoglycans in peak C contain a high carbohydrate-to-protein ratio.

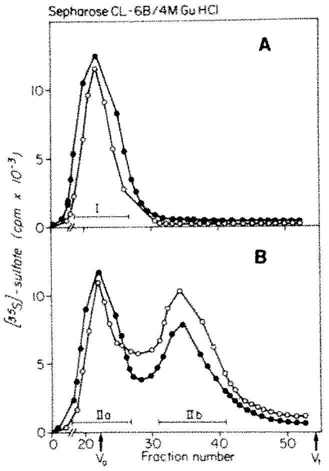

Fig 6. Preparative Sepharose CL-6B elution profiles of the void volume peaks (MI, CI) and included peaks (MII, CII) obtained from chromatography on Sepharose CL-4B (see Fig 5). Chromatography was performed using dissociative conditions (4 mol/L GuHCl). Bars represent those fractions that were pooled for further analysis.

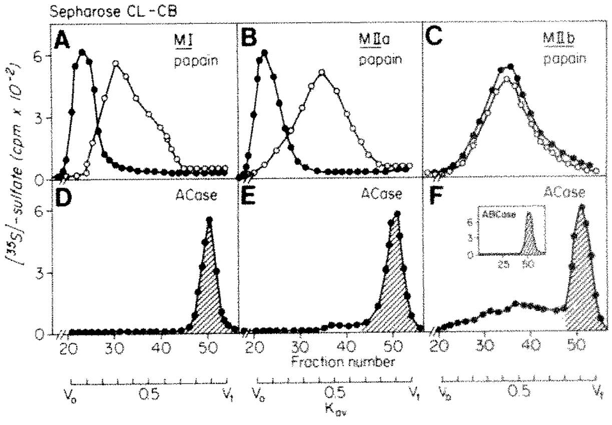

Fig 7. Sepharose CL-6B elution profiles of ^{35}S-sulfate labeled macromolecules present in medium peaks, MI, MIIa, and MIIb before (solid circles) and after (open circles) papain (panels A, B, C) and chondroitiniase AC treatment (panel D, E, F). The inset in panel F is the Sepharose CL-6B profile after MIIb had been treated with chondroitinase ABC.

Peak C from both the medium and cell layer extracts was further analyzed by chromatography on Sepharose CL-4B in 4 mol/L GuHCl. Both culture compartments gave similar chromatographic profiles (Fig 5). A single, well-defined peak (MI; CI) eluted in the void volume of the column, followed by a broad second peak that eluted at a position of K_{av} = ~0.3 (MII; CII). Major peak fractions (MI, MII, CI, CII) were pooled as indicated in Fig 5 and further analyzed by chromatography on Sepharose CL-6B using 4 mol/L GuHCl as the eluting buffer. MI and CI eluted as well-defined peaks in the void volume (Fig 6), while MII and CII eluted as two peaks on Sepharose 6B, one eluting in the void volume of the column (MIIa; CIIa) and the other eluting at a K_{av} = 0.39 (MIIb; CIIb). Each of the identified peaks were further analyzed as to their susceptibility to papain and chondroitinase AC digestion. Papain treatment of MI, MIIa,

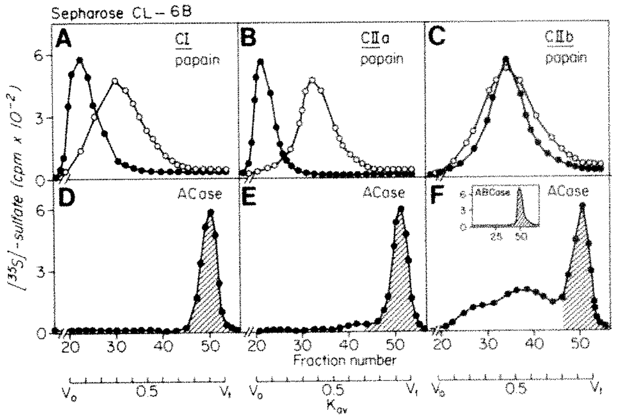

Fig 8. Sepharose CL-6B elution profiles of ^{35}S-labeled macromolecules present in cell layer peaks CI, CIIa, and CIIb before (solid circles) and after (open circles) papain (panel A, B, C) and chondroitinase AC (panel D, E, F) treatment. The inset in panel F is the Sepharose CL-6B profile afer CIIb had been treated with chondroitinase ABC.

CI, and CIIa shifted these peaks from the void position on Sepharose CL-6B to an elution position of $K_{av} = 0.38$ to 0.40 (Figs 7 and 8). Similar results were obtained when chains were released by alkaline borohydride. These results indicate that each of these peaks represent an intact proteoglycan with glycosaminoglycan chains of nearly equal size (~38,000).[29] On the other hand, papain treatment of MIIb and CIIb did not shift the elution position of these peaks, indicating that these peaks contain either free glycosaminoglycan chains or a single chain attached to a small peptide.[23]

Digestion of MI and CI with chondroitinase AC resulted in greater than 98% degradation of each peak of disaccharides as determined by Sepharose CL-6B chromatography (Figs 7D and 8D). Peak MIIa was also nearly totally (97%) susceptible to chondroitinase AC digestion, whereas CIIa was ~86% susceptible (Figs 7E and 8E). Peaks MIIb and CIIb contained a much higher percentage (~40% of the total label) of partially degraded material following chondroitinase AC treatment that eluted ahead of the disaccharide peak (Figs 7F and 8F). Since this material was totally

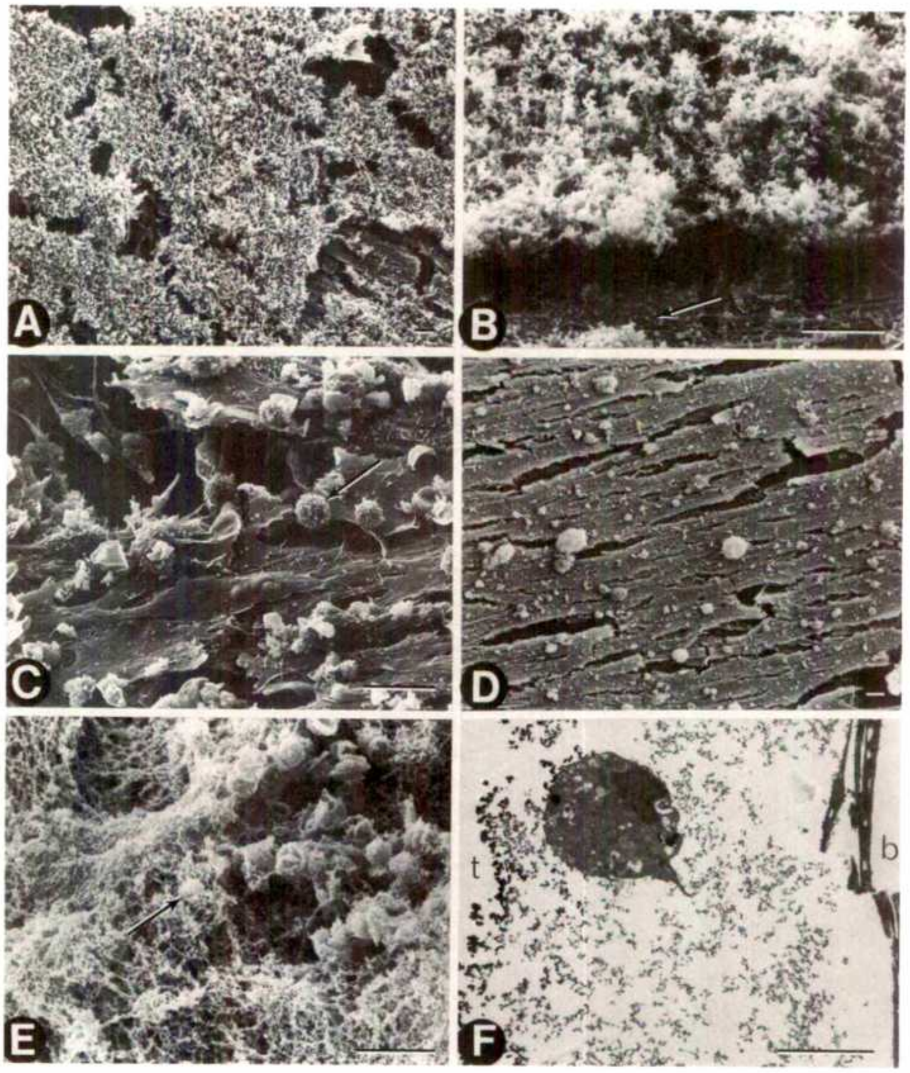

Fig 9. (A) Low-magnification scanning electron micrograph of a long-term bone marrow culture (4 weeks) fixed and processed in the presence of ruthenium red. A mat of flufflike material covers a layer of flattened stromal cells. Bar = 10 μm (original magnification ×300); current magnification ×207. (B) A higher magnification of this matted material reveals a fibrillar meshwork extending a considerable distance above the adherent cells. (arrow) Original magnification ×1650; current magnification ×1138. Bar = 10 μm. (C) This scanning electron micrograph was taken of a comparable long-term bone marrow culture that was prepared for electron microscopy in the absence of ruthenium red. No matlike substance is seen. Numerous hematopoietic cells can be seen associated with the stromal layer of adherent cells (arrow). Original magnification ×1290; current magnification ×890. Bar = 10 μm. (D) This scanning electron micrograph was taken from a long-term bone marrow culture that had been pretreated with chondroitinase ABC prior to processing for electron microscopy in the presence of ruthenium red. The enzyme treatment completely eliminated the mat of surface material lying above the adherent cells. Original magnification ×360; current magnification ×248. Bar = 10 μm. (E) A high magnification of this flocculent material reveals a number of rounded cells enmeshed in the material preserved with ruthenium red (arrow). Original magnification ×1,500; current magnification ×1035. Bar = 10 μm. (F) Transmission electron microscopy of SEM preparations reveals that the round cells are early hematopoietic cells. Dark staining at the left of the micrograph is the gold coating on the surface of the mat. Cells on the right side of the micrograph are those present within the adherent layer (b, culture dish). Original magnification ×3,750; current magnification ×2,587. Bar = 5 μm.

degraded to disaccharide by chondroitinase ABC treatment (inset, Figs 7F and 8F), peaks MIIb and CIIb must be enriched in iduronic acid and considered to contain dermatan sulfate.

Electron Microscopy

Cultures fixed in the presence of ruthenium red and processed for scanning electron microscopy revealed a mat of flocculent material lying upon portions of layered and flattened adherent cells (Figs 9A and B). This material was absent if ruthenium red was omitted from the fixative (Fig 9C) or if the cultures were treated with chondroitinase ABC prior to rutheniun red staining (Fig 9D). Both scanning and transmission electron microscopy demonstrated the presence of early hematopoietic cells throughout this flocculent material (Figs 9E and F). The ruthenium red–positive layer extended a distance of at least 25 μm above the cells and consisted of aggregates of dense granules frequently joined by thin 5-nm diameter filaments (Figs 10A and B). The thin

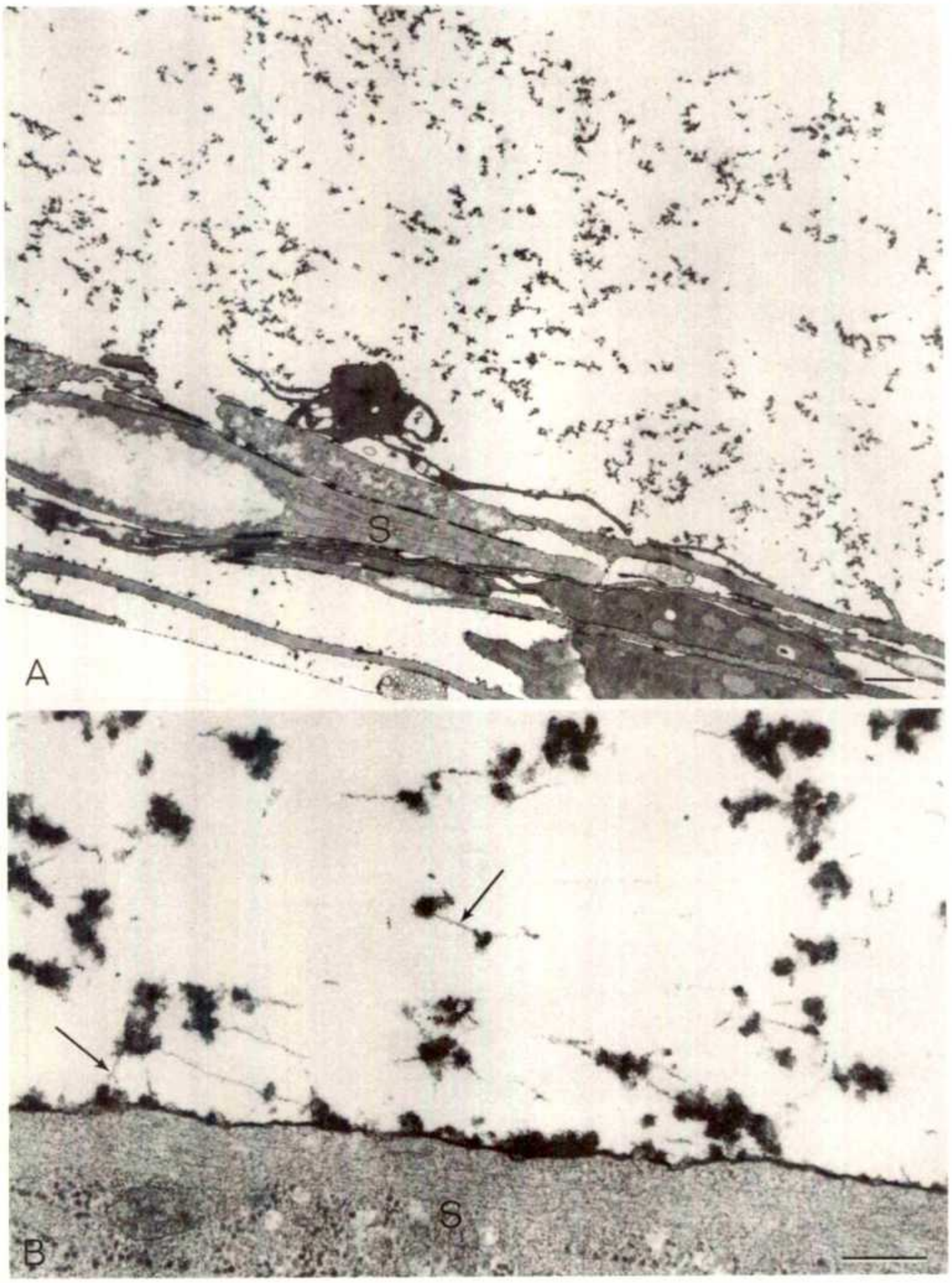

Fig 10. **(A) Transmission electron microscopy of cross sections of these cultures prepared in the presence of ruthenium red reveals an extensive layer of granular material lying above a multilayer of adherent cells (S). Original magnification ×9,600; current magnification ×6,720. Bar = 1.0 μm. (B) Higher magnification of this substance reveals clumps of dark-staining granules occasionally connected to one another via thin 5-nm threads (arrows). The surface of the adherent cells (S) also stains intensely with ruthenium red. Original magnification ×60,000; current magnification ×42,000. Bar = 0.25 μm.**

filaments frequently associated with the surface of flattened cells in the multilayer (Fig 10B). Prominent ruthenium red staining of the surface of the adherent cells as well as the hematopoietic cells was a consistent feature of these cultures (Figs 10 and 11). Frequently, areas of contact between early hematopoietic cells and adherent cells were stained with ruthenium red (Fig 11). Ruthenium red–positive granules were also distributed among dark-staining fibrils between adjacent cells of the multilayer (Fig 12). These dark-staining fibrils resisted elimination by chondroitinase ABC treatment whereas the granules were totally removed by this enzyme treatment. The fibrils resembled fibrils shown to contain fibronectin[34] which are enriched in long-term bone marrow cultures[35] and may contain heparan sulfate proteoglycan, which is known to bind to fibronectin.[36]

The ability of chondroitinase ABC treatment to remove the majority of the proteoglycans present in the cell layer was confirmed when prelabeled cultures were incubated with the enzyme. Chondroitinase ABC treatment removed 70% of the ^{35}S-sulfate–labeled macromolecules present in the cell layer whereas buffer treatment alone removed 10%. The enzyme treatment did not remove adherent cells, and the conditions used to demonstrate the loss of ruthenium red–positive granules paralleled those previously described.

DISCUSSION

The major sulfated proteoglycans synthesized by human long-term bone marrow cultures can be separated into at least three families based on their relative charge, hydrodynamic size, and/or glycosaminoglycan composition. A large chondroitin sulfate proteoglycan (CSPG) containing glycosaminoglycan chains of ~38,000 mol wt predominates in both the medium and cell layer compartments of these cultures. A similar large CSPG has been observed in a number of other culture systems including arterial smooth muscle,[22,23] glial cells,[37,38] skin, and lung fibroblasts.[39,40] These findings indicate that this class of proteoglycan can be synthesized by a variety of cell types. The other major proteoglycan present in these cultures is hydrodynamically smaller and contains glycosaminoglycan chains of nearly equal size to those of the larger CSPG, but unlike the CSPG, the glycosaminoglycan chains contain a small percentage of iduronic acid residues (~10%) as part of the total uronic acid present in the molecule. It is uncertain at this point whether the iduronate in MIIa and CIIa might originate from incomplete separation from MIIb and CIIb, which are rich in iduronate. However, similar low iduronate acid–containing proteoglycans have been described in a variety of tissues[41,42] and cultures of various types of cells.[43,44] Interestingly, a papain-resistant, sulfate-labeled dermatan sulfate peak that contains a higher percentage of iduronate (~40%) is found in both the cell layer and medium compartments. The finding that this peak does not shift upon chromatography in Sepharose CL-6B following papain treatment indicates that the peak contains either free glycosaminoglycan chains or single chains attached to a short peptide.[27,45] Similar papain-resistant peaks have been observed in cell layer extracts of a variety of cells in culture,[23,27,45] and these peaks have been interpreted to represent intracellular degradation products of proteoglycans present within lysosomes.[45] However, a similar molecule appears to gain access to the medium compartment in bone marrow cultures. It is uncertain at this point whether this media-derived, papain-resistant material represents degraded proteoglycan released from the cells or extracellular degradation products. Studies are currently being conducted to investigate the source of this low–molecular weight material.

Although the majority of ^{35}S-sulfate–labeled material present in both the medium and the cell layer compartment in human bone marrow cultures contains chondroitin and/or dermatan sulfate, a small amount of ^{35}S-sulfate activity (~5%) is present in nitrous acid–sensitive material, indicating the presence of heparan sulfate. This material was present in both medium and cell layer fractions and could be effectively separated from the chondroitin- and dermatan sulfate–containing molecules by ion-exchange chromatography. The finding that the heparan sulfate–containing material elutes before the chondroitin-dermatan sulfate–contain-

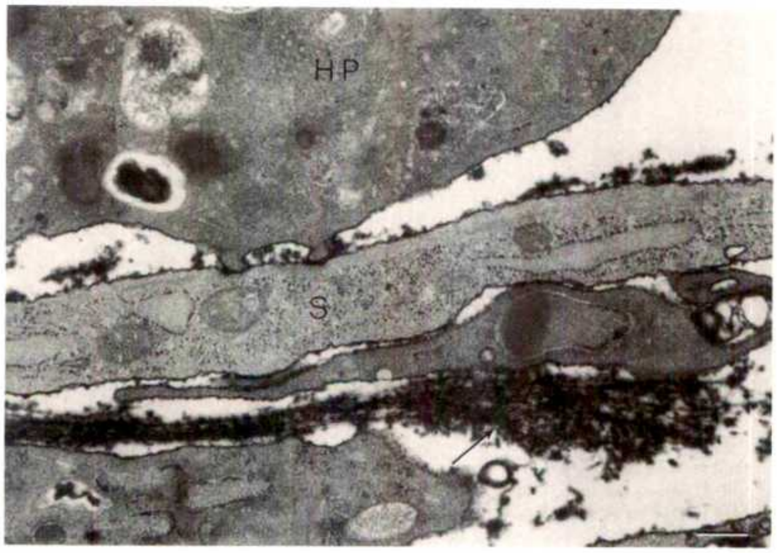

Fig 11. Occasionally, hematopoietic cells (HP) can be seen to contact adherent stromal cells (S) via knobby extensions of their cytoplasm in regions enriched in ruthenium red staining. Dark staining filamentous material (arrow) is also prominent between adherent cells in the multilayer. Original magnification ×42,000; current magnification ×24,780. Bar = 0.25 μm.

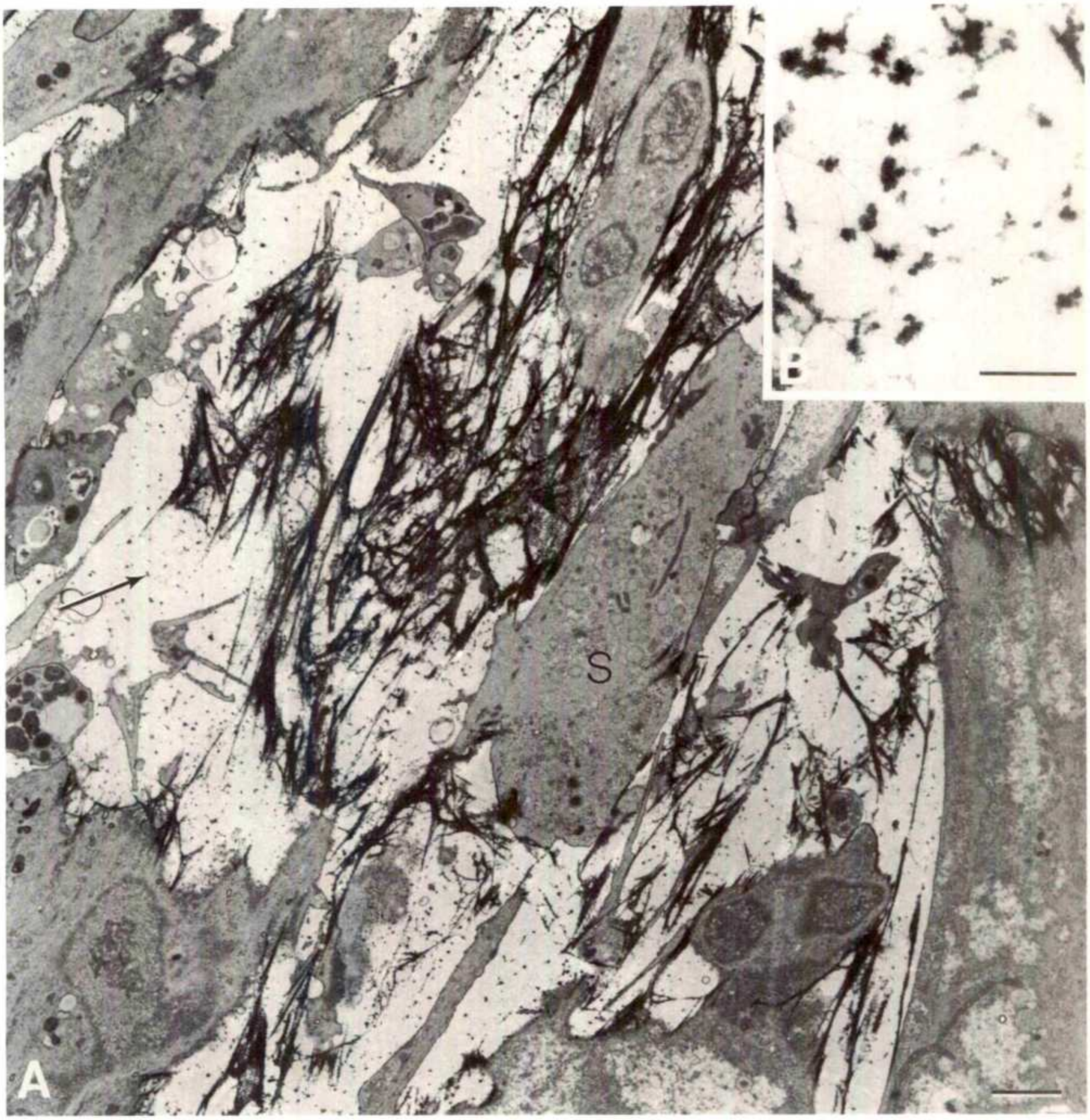

Fig 12. (A) This electron micrograph was taken from a longitudinal section through the multilayer of adherent cells (S) fixed and processed in the presence of ruthenium red. Numerous dark-staining fibrils and a large number of ruthenium red–positive granules (arrows) are present throughout the intercellular space. A high magnification (B) of the granules reveals that many of them are aggregated and connected via thin 5-nm threads. (A), original magnification ×4,320; current magnification ×4,190. Bar = 2.5 μm. (B), original magnification ×27,000; current magnification ×26,196. Bar = 0.25 μm.

ing material on the DEAE ion-exchange column indicates that the heparan sulfate present in these cultures is less charged than the chondroitin–dermatan sulfate.

The structural and compositional characteristics of the proteoglycans synthesized in the human bone marrow cultures resembles those proteoglycans synthesized by arterial smooth muscle cells in vitro.[22,23] These similarities include similar ratios of HSPG to CSPG/DSPG, identical behavior on ion-exchange and molecular-sizing columns, similar distribution in a dissociative CsCl density gradient, and similar glycosaminoglycan composition and chain size. In addition, like proteoglycans from arterial smooth muscle cells, some of the proteoglycans present exist in aggregate form (data not shown). Although long-term bone marrow cultures contain a mixture of cell types including endothelial cells, fibroblasts, macrophages, adipocytes, and hematopoietic stem cells,[46] recent studies indicate that approximately 50% of the adherent cells in the human long-term bone marrow cultures react with monoclonal antibodies specific for smooth muscle cells.[46] At present, it is not clear whether all proteoglycans synthesized and secreted in the bone marrow cultures originate from smooth muscle–like cells since other cell types present such as endothelial cells,[47] fibroblasts,[39,40] monocytes,[48] and granulocytes[49–50] are all capable of synthesizing and secreting proteoglycans. However, recent studies using mouse marrow cultures indicate that the adherent cells and not the nonadherent hemopoietic cells were the major source of glycosaminoglycans and that the predominant glycosaminoglycan was heparan sulfate.[51]

The presence of a matlike arrangement of ruthenium red positive granules and filaments above the monolayer of adherent cells is unique to this culture system, since similar structures have not been observed within cultures of other cell types. The susceptibility of this material to chondroitinase ABC and the granular texture suggests that this layer is enriched in proteoglycan. The finding that hemopoietic stem cells are enmeshed in the proteoglycan-rich layer indicates close association of stem cells with a proteoglycan-rich matrix. Whether this matrix is critical in promoting stem cell interaction with the adherent cells and subsequent hematopoiesis remains to be determined. It will be of interest to examine the nature of proteoglycans present in marrow cultures which exhibit suppressed or defective hematopoiesis such as cultures derived from Sl/Sld mice, or patients with leukemia or aplastic anemia.

In conclusion, proteoglycans have been implicated in morphogenetic events for some time,[8] but the exact nature of their influence has remained enigmatic. A key question that still remains is whether these molecules function merely to stabilize the differentiated state and create the proper microenvironment for morphogenetic events to proceed or act as inductive elements directly affecting those cellular events necessary for differentiation. The development of an in vitro system which supports differentiation (ie, hemopoiesis) and which possesses macromolecules confined to the microenvironment not only offers the opportunity to address this question but also may provide insight into the mechanisms by which specific proteoglycans influence differentiation.

ACKNOWLEDGMENT

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REFERENCES

1. Wolf N: The haemopoietic microenvironment. Clin Haematol 8:469, 1979

2. Dexter TM: Stromal cell associated haemopoiesis. J Cell Physiol 1:87, 1982 (suppl)

3. Dexter TM: Allen TD, Lajtha LG: Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335, 1977

4. Dexter TM, Spooncer E, Tokoz D, Lajtha LG: The role of cells and their products in the regulation of in vitro stem cell proliferation and granulocyte development. J Supramol Struct 13:513, 1980

5. Gartner S, Kaplan HS: Long term culture of human bone marrow cells. Proc Natl Acad Sci USA 77:4756, 1980

6. Allen TD: Haemopoietic microenvironments in vitro: Ultrastructural aspects, in Porter R, Whelan J (eds): Microenvironments in Haemopoietic and Lymphoid Differentiation. Ciba Foundation Symposium 84. London, Pitman, 1981, p 38

7. Hascall VC, Hascall GK: Proteoglycans, in Hay E (ed): Cell Biology of Extracellular Matrix. New York, Plenum, 1981, p 39

8. Toole BP: Glycosaminoglycans in morphogenesis, in Hay E (ed): Cell Biology of Extracellular Matrix. New York, Plenum, 1981, pp 259

9. Pearson H: Marrow hypoplasia in anorexia nervosa. J Pediatr 71:211, 1967

10. Tavassoli M, Eastlund DT, Yam LT, Neiman RS, Finkel H: Gelatinous transformation of bone marrow in prolonged self-induced starvation. Scand J Haematol 16:311, 1976

11. McCuskey RS, Meinke HA: Studies of the hemopoietic microenvironment. III. Differences in the splenic microvascular system and stroma between Sl/Sl_d and W/W^v anemic mice. Am J Anat 137:187, 1973

12. Schrock LM, Judd JT, Meinke HA, McCuskey RS: Differences in concentrations of acid mucopolysaccharides between spleens of normal and polycythemic CF_1 mice. Pro Soc Exp Biol Med 144:593, 1973

13. Pleomacher RE, Vant Hull E, Van Soest PL: Studies of the hemopoietic microenvironments: Effects of acid mucopolysaccharides and dextran sulfate on erythroid and granuloid differentiation in vitro. Exp Hematol 6:311, 1978

14. Vannucchi S, Fibbi G, Cella C, Del Rosso M, Cappelletti R, Chiarugi V: Cell surface glycosaminoglycans in normal and leukemic leukocytes. Cell Differ 9:71, 1980

15. Del Rosso M, Cappelletti R, Dini G, Fibbi G, Vannucchi S, Chiarugi V, Guazzelli C: Involvement of glycosaminoglycans in detachment of early myeloid precursors from bone marrow stromal cells. Biochim Biophys Acta 676:129, 1981

16. Spooncer E, Gallagher JT, Kriza F, Dexter TM: Regulation of hemopoiesis in long term bone marrow cultures. IV. Glycosaminoglycan synthesis and the stimulation of hemopoiesis by β-D-xylosides. J Cell Biol 96:510, 1983

17. Schwartz NB: Regulation of chondroitin sulfate synthesis. Effect of β-xylosides on synthesis of chondroitin sulfate proteoglycan, chondroitin sulfate chains and core protein. J Biol Chem 252:6316, 1977

18. Schwartz NB, Galligani L, Ho P-L, Dorfman A: Stimulation of synthesis of free chondroitin sulfate chains by β-D-xylosides in cultured cells. Proc Natl Acad Sci USA 71:4047, 1974

19. Galligani L, Hapwood L, Schwartz NB, Dorfman A: Stimulation of synthesis of free chondroitin sulfate chains by β-D-xylosides in cultured cells. J Biol Chem 250:5400, 1975

20. Thompson H, Spooner B: Proteoglycan and glycosaminoglycan synthesis in embryonic mouse salivary glands: Effects of β-D-xyloside, an inhibitor of branching morphogenesis. J Cell Biol 96:1443, 1983

21. Singer JW, Fialkow PJ, Dow LW, Ernst C, Steinmann L: Unicellular or multicellular origin of human granulocyte-macrophage colonies in vitro. Blood 54:1395, 1979

22. Wight, TN, Hascall VC: Proteoglycans in primate arteries. III. Characterization of the proteoglycans synthesized by arterial smooth muscle cells in culture. J Cell Biol 96:167, 1983

23. Chang Y, Yanagishita M, Hascall VC, Wight TN: Proteoglycans synthesized by smooth muscle cells derived from monkey (*Macaca nemistrina*) aorta. J Biol Chem 258:5679, 1983

24. Ohya T, Kaneko Y: Novel hyaluronidase from *Streptomyces*. Biochim Biophys Acta 198:607, 1970

25. Cifonelli J: Reaction of heparatin sulfate with nitrous acid. Carbohydr Res 8:233, 1963

26. Saito H, Yamagata T, Suzuki S: Enzymatic methods for the determination of small quantities of isomeric chondroitin sulfates. J Biol Chem 243:1536, 1968

27. Yanagashita M, Hascall VC: Proteoglycans synthesized by rat ovarian granulosa cells in culture. Isolation, fractionation and characterization of proteoglycans associated with the cell layer. J Biol Chem 259:10260, 1984

28. Hascall VC, Heinegard D: Aggregation of cartilage proteoglycans. I. Role of hyaluronic acid. J Biol Chem 249:4232, 1974

29. Wasteson A: A method for the determination of the molecular weight and molecular weight distribution of chondroitin sulfate. J Chromatogr 59:87, 1971

30. Wight TN: Differences in the synthesis and secretion of sulfated glycosaminoglycans by aorta explant monolayers cultured from atherosclerosis-susceptible and resistant pigeons. Am J Pathol 101:127, 1980

31. Chen K, Wight TN: Proteoglycans in arterial smooth muscle cell cultures: An ultrastructural histochemical analysis. J Histochem Cytochem 32:347, 1984

32. Schmidt RA, Glomset JA, Wight TN, Habenicht A, Ross R: A study of the influence of mevalonic acid and its metabolites on the morphology of Swiss 3T3 cells. J Cell Biol 95:144, 1982

33. Oike Y, Kimata K, Shinomura T, Suzukie S: Proteinase activity in chondroitin lyase (chondroitinase) and endo-β-D-galactosidase (keratanase) preparations and a method to abolish their effect on proteoglycan. Biochem J 191:203, 1980

34. Chen L, Murray A, Segal RA, Bushnell A, Walsh ML: Studies on intercellular LETS glycoprotein matrices. Cell 14:377, 1978

35. Reincke U, Hsieh P, Mauch P, Hellman S, Chen LB: Cell types associated with fibronectin in long term mouse bone marrow cultures. J Histochem Cytochem 30:235, 1982

36. Rouslahti E, Engvall E: Complexing of fibronectin, glycosaminoglycans and collagen. Biochim Biophys Acta 631:350, 1980

37. Norling B, Glimelius B, Westermark B, Wasteson A: A chondroitin sulfate proteoglycan from human cultured glial cell aggregates with hyaluronic acid. Biochem Biophys Res Commun 84:914, 1978

38. Norling B, Glimelius B, Westermark B, Wasteson A: A chondroitin sulfate proteoglycan from human cultured glioma cells. Biochem J 221:845, 1984

39. Cöster L, Carlstedt I, Mälmstrom A: Isolation of [^{35}S] and [^{3}H] labelled proteoglycans from cultures of human embryonic skin fibroblasts. Biochem J 183:669, 1979

40. Vogel KG, Peterson DW: Extracellular, surface and intracellular proteoglycans produced by human embryo lung fibroblasts in culture (IMR-90). J Biol Chem 256:13235, 1981

41. Kapoor R, Phelps CF, Coster L, Fransson LA: Bovine aortic chondroitin sulfate–dermatan sulfate containing proteoglycans. Biochem J 197:259, 1981

42. Pearson CH, Gibson GF: Proteoglycans of bovine peridontal ligament and skin occurrence of different hybrid-sulfated galactosaminoglycans in distinct proteoglycans. Biochem J 201:27, 1982

43. Gallagher JT, Gasiunas N, Schor SL: Specific association of iduronic acid–rich dermatan sulfate with the extracellular matrix of human skin fibroblasts cultured on collagen gels. Biochem J 215:107, 1983

44. Lark M, Wight TN: Modulation of proteoglycan metabolism by aortic smooth muscle cells grown on collagen gels. J Cell Biol 99:174, 1984

45. Yanagashita M, Hascall VC: Metabolism of proteoglycans in rat ovarian granulosa cell culture. Multiple intracellular degradative pathways and the effect of chloroquine. J Biol Chem 259:10270, 1984

46. Singer JW, Keating A, Cattner JM: 1984: Evidence for a stem cell common to hematopoiesis and its in vitro microenvironment. Leuk Res 8:535, 1984

47. Oohira A, Wight TN, Bornstein P: Sulfated proteoglycans synthesized by vascular endothelial cells in culture. J Biol Chem 258:2014, 1983

48. Levitt D, Ho PL: Induction of chondroitin sulfate proteoglycan synthesis and secretion in lymphocytes and monocytes. J Cell Biol 97:351, 1983

49. Orenstein NS, Galli SJ, Dvorak AM, Silbert JE, Dvorak HF: Sulfated glycosaminoglycan of guinea pig basophilic leukocytes. J Immun 121:586, 1978

50. Olssen I, Gardell S: Biosynthesis of glycosaminoglycans in leukocytes. Biochim Biophys Acta 237:203, 1971

51. Gallagher JT, Spooncer E, Dexter TN: Role of extracellular matrix in haemopoiesis. I. Synthesis of glycosaminoglycans by mouse bone marrow cell cultures. J Cell Sci 63:155, 1983

Antigenic Determinant in Human Coagulation Factor IX: Immunological Screening and DNA Sequence Analysis of Recombinant Phage Map a Monoclonal Antibody to Residues 111 Through 132 of the Zymogen

By Royal McGraw, Dan Frazier, Mark de Serres, Howard Reisner, and Darrell Stafford

As an approach to the study of structure–function relationships in the normal and defective forms of human coagulation factor IX, we have begun to develop a series of monoclonal antibodies against specific sites on the protein. Zymogen and activated forms of normal factor IX were used initially as antigen for the preparation of monoclonal antibodies. Recombinant phage were prepared by cloning small (50- to 500-nucleotide) random DNA fragments from the coding region of a factor IX cDNA clone into the expression vector λ gt11. Immunological screening of these recombinants with mixtures of monoclonal antibodies identified several immunoreactive phage. Further analysis showed that the monoclonal antibody designated IX-30 was generating the positive signals at a frequency of ~1/2,500 recombinants. Subcloning and sequence analysis of the inserted DNA in the immunoreactive phage revealed overlapping in-frame insertions, from which it could be inferred that the site in factor IX recognized by IX-30 is confined to residues 111 through 132 in the light chain. Similar mapping with other monoclonal antibodies should provide additional probes for the protein structure of human factor IX.

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FACTOR IX is the plasma protein that is absent or defective in individuals with the hereditary X-linked bleeding disorder known as hemophilia B or Christmas disease. Among patients with dysfunctional factor IX, considerable heterogeneity has been demonstrated both with regard to the biochemical characteristics of the abnormal protein and to the severity of the associated bleeding.[1] As an essential component of the coagulation cascade, the normal activity of factor IX requires appropriate interactions with various activators, cofactors, and substrate(s). Although these molecular events are not entirely understood, we expect that mutations altering any of these interactions may be sufficient to cause hemophilia.

To investigate further the structure–function relationships in both normal and defective factor IX molecules, we have begun to identify monoclonal antibodies for use as probes against specific sites on the protein. To locate epitopes on factor IX, we have combined immunological and recombinant DNA approaches by use of the expression vector λ gt11. This bacteriophage vector was originally developed by Young and Davis[2] to facilitate the cloning of protein-coding genes through use of antibody probes to detect expressed proteins. The system has further been used as a source of specific antigen to raise new antisera against the product of a cloned gene.[3] Although information concerning the nature of the antigenic site is inherent in these procedures, Nunberg and co-workers have refined the method to allow a rather precise mapping of epitopes recognized by monoclonal antibodies.[4]

We used the strategy of Nunberg and co-workers to generate a population of recombinant phage containing small segments of the human factor IX gene. Screening these recombinants with nine monoclonal antibodies raised against human factor IX identified several immunoreactive phage. Subsequent screenings indicated that only one of the monoclonals (designated IX-30) yielded positive clones. Sequence analysis of the cloned DNA segments then revealed overlapping in-frame insertions from which boundaries for the antigenic site could be inferred. We conclude that the antigenic site recognized by IX-30 is confined to amino acid residues 111 through 132 in the light chain of human factor IX. This segment of the factor IX protein represents a region of local hydrophilicity and has interesting homologies with several other coagulation proteins and growth factors.

MATERIALS AND METHODS

Monoclonal antibodies. Balb/c mice were immunized intraperitoneally with a mixtue of zymogen and activated forms of human factor IX (with adjuvants) over a six-month period at triweekly intervals. After four to six months of rest, they were boosted twice, with the last injection being given five days prior to fusion. Splenocytes were fused with P3X63-Ag8.653 cells. Fusion methodology and screening protocols were similar to those previously described.[5,6] For direct enzyme-linked immunosorbent assays (ELISAs), the wells of polyvinyl chloride microtiter plates were coated with 75 μL of factor IX or factor IXa solutions containing 25 μg/mL of protein. Plates were blocked with an excess of bovine serum albumin (BSA) prior to use. Assays to determine the influence of divalent cations were done with either 5 mmol/L of Ca^{2+} or 10 mmol/L of EDTA in all steps. Monoclonality was achieved by recloning at limiting dilution and was verified by isoelectric focusing of the antibody preparations.

Nine of these monoclonal reagents, showing ELISA titers for factor IX between 1×10^3 and 1×10^6, were used for screening the recombinant phage library. Four of these showed >1,000-fold enhancement of binding in the presence of Ca^{2+}, whereas three showed significant reactivity in the absence of divalent cations.

Inhibition assays and immunoblots with monoclonal reagent IX-30. ELISA-based inhibition assays were done as above except that a 1.0 μg/mL solution of factor IX was used to coat the plates. Dilutions (0.5×10^5 or 1.0×10^5) of IX-30 ascites fluid were preincubated with dilutions of test protein or buffer for two hours at 37 °C and were then added to the assay plates. Test proteins included factor IX, factor IXa, and factor X (provided by Drs D.M. Monroe and H.R. Roberts), bovine factor IX (provided by Dr A. Thompson), and human protein C (provided by Dr W. Kisiel).

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Samples of the test proteins (10 μg) were analyzed by electrophoresis on discontinuous 10% sodium dodecyl sulfate (SDS) gels as described by Laemmli.[7] The proteins were electrophoretically transferred to cellulose nitrate sheets (Bio-Rad, Richmond, Calif) according to manufacturer's recommendations. The sheets were incubated with a 1/100 dilution of IX-30 ascites, followed by peroxidase-labeled goat anti-mouse immunoglobulin (Jackson Laboratories, Avondale, Penn). Horseradish peroxidase (HRP) reagent (Bio-Rad) was then used to visualize immunoreactive bands. Proteins in gels were also directly visualized by staining with Coomassie brilliant blue.

Recombinant phage. Most DNA manipulations were done by established methods.[8] The starting material was a 1.3-kilobase (kb) *Hae*III restriction fragment from the cloned human factor IX cDNA.[9] This fragment contains all of the coding sequences for the zymogen form of factor IX. As a positive control for the immunological screening, this fragment was cloned directly into the *Eco*R1 site of the phage vector λ gt11 using 12-mer *Eco*R1 linkers. Inserted with the appropriate orientation, this would give a correct reading frame for expression. The 1.3-kb fragment was also used to prepare a library of random, small insertions in λ gt11. This was done essentially as described by Nunberg and co-workers,[4] with minor variations. The cDNA fragment (~3 μg) was partially degraded with DNAse I (deoxyribonuclease I) to give fragments predominantly in the size range of 50 to 500 base pairs (bp). The ends of these fragments were made blunt with T4 DNA Polymerase. *Eco*R1 linkers were added with T4 DNA ligase and, following digestion with *Eco*R1, this material was fractionated by electrophoresis in a 1.5% gel of low-melting-point agarose. A gel slice containing fragments in the desired size range was excised and melted. The DNA was then purified by repeated phenol extractions and ethanol precipitation.

Vector DNA was purified from liquid culture by conventional means. This DNA was then self-ligated, cleaved with *Eco*R1, and treated with calf intestinal alkaline phosphatase to prevent religation of the arms. The random factor IX fragments were then ligated with vector and packaged into phage particles using extracts prepared according to Grosveld et al.[10]

Immunological screening. The recombinant phage were screened for factor IX antigens essentially as described by Nunberg and co-workers.[4] In brief, the following steps were involved: (a) phage were plated at a density of several thousand plaques per 150-mm dish; (b) a few hours after plating, the phage were covered with isopropyl β-D-thiogalactoside (IPTG)-impregnated nitrocellulose filters, and incubation was continued overnight (IPTG is a galactose analog and a potent inducer of bacterial β-galactosidase gene expression); (c) the filters were removed and rinsed, and nonspecific binding was blocked with bovine serum albumin (BSA); (d) filters were rinsed, incubated with monoclonal antibodies overnight at 4 °C; (e) filters were washed, incubated with affinity-purified rabbit anti-mouse serum; (f) filters were washed, incubated with ^{125}I-labeled Protein A, and washed again; and (g) filters were autoradiographed. Positive phage were picked, replated at lower density, and rescreened until pure.

Sequence analysis. Positive phage were grown in 100-mL liquid cultures to obtain sufficient DNA for analysis of the inserted factor IX segments. Phage DNA was cut with *Eco*RI and fractionated by agarose (0.7%) gel electrophoresis. Insert fragments were electroeluted, purified, and recloned into the *Eco*RI site of M13 mp8 for sequence analysis. These recloned segments were then sequenced by the dideoxy method,[11,12] as modified by McGraw,[13] using end-labeled universal primer as the source of radiolabel.

The relevant protein sequences were inferred from DNA sequence. The hydrophilicity/hydropathy profile was generated by a computer program based on the method of Kyte and Doolittle.[14]

RESULTS

Immunological screening. As shown in Fig 1a, ~50% of the plaques resulting from ligation of the 1.3-kb factor IX cDNA segment into λ gt11 gave positive signals. This was consistent with the expectation that only half of the insertions would be oriented appropriately for expression. This result indicated that our screening procedure was correct, and that at least one of the monoclonals (three were used as a mixture in this experiment) was capable of recognizing the bacterially synthesized factor IX polypeptide.

Subsequent screening with individual monoclonal sera (data not shown) indicated that only one of the antibodies, designated IX-30, gave positive signals. The eight others gave negative results both with the control clone and with the fragment library. When IX-30 was used to screen the fragment library, positive plaques were identified at a frequency of ~1/2,500 recombinants. A positive signal obtained on primary screening of the fragment library is shown in Figure 1b. Three such clones were plaque-purified and analyzed further.

Sequence analysis. The first immunoreactive recombinant to be fully characterized had the designation Gor9. The inserted fragment in this phage was 169 nucleotides in length and was shown by DNA sequencing to code for the 37

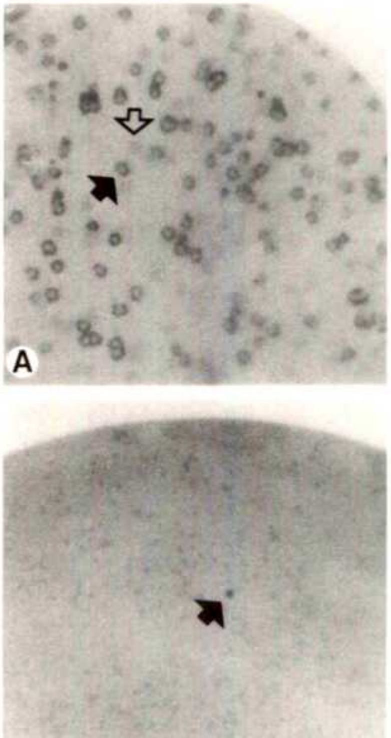

Fig 1. Immunological screening of recombinant phage. Each panel represents a 5 × 5 cm^2 section of an autoradiograph. (A) Positive control clones prepared with the 1,300-base pair cDNA fragment in λ gt11. Dark arrow, immunoreactive plaque; light arrow, negative plaque, presumably containing the insert in the opposite orientation. (B) Primary screening of the fragment library. Arrow points to a single positive plaque.

residues from Pro^{74} through Cys^{132} in the light-chain region of factor IX. Sequence patterns were obtained from both ends of the insert by recloning the isolated fragment into the sequencing vector M13mp8. Each end had an *Eco*R1 half-linker as expected from the cloning regimen, and the end representing the amino-terminus of the encoded peptide had the appropriate reading frame for expression when placed in the *Eco*R1 site of λ gt11.

Two other positive clones from the fragment library were characterized similarly. The results are summarized in Fig 2. The clone designated Gor1 contained a 222-nucleotide fragment encoding the 74 residues from Cys^{111} to Gly^{184}. This sequence covers 35 residues of the light chain, the entire activation peptide region (also 35 residues), and 4 residues of the heavy chain. Because this sequence overlaps one end of the sequence in Gor9, it could be inferred that the antigenic determinant recognized by IX-30 lies in the region of overlap. The overlapping region is confined to the light chain, and as shown in Fig 2, includes the 22 residues extending from Cys^{111} through Cys^{132}.

The third positive clone, designated Gor7, was found to encode 196 factor-IX amino acid residues, including four residues of the leader peptide, all of the light chain, the activation peptide, and 12 residues of the heavy chain (Arg^{-4}–Phe^{192}). This clone spans all sequences of both Gor9 and Gor1. As such, it imposes no further restrictions on the extent of the antigenic site.

Hydrophilicity profile. Consistent with the prediction of Hopp and Woods,[15] the peptide sequence mapped with IX-30 represents a region of local hydrophilicity in the factor IX protein. The result of a computer analysis demonstrating this feature of the antigenic region is presented in Fig 3. Because the seven residues at the carboxyl end of the mapped region are clearly hydropathic in character, it seems likely that the essential antigenic determinant may be contained entirely within the preceding 10 or 12 residues.

Immunoreactivity of IX-30. The monoclonal antibody IX-30 appeared to have a limited reactivity with the human factor X preparations as judged by ELISA. Inhibition assays also suggested that both the factor X and protein C preparations were able to inhibit the binding of IX-30 to human factor IX slightly. The extent of this inhibition appeared greater with factor X than with protein C, but was equivalent to no more than a few percent of the factor IX reactivity in either case (~3% and 1%, respectively). These results are shown in Fig 4. Also shown are results obtained with bovine factor IX and activated human factor IX, both of which proved to be highly effective competitors for the binding of IX-30 to human factor IX.

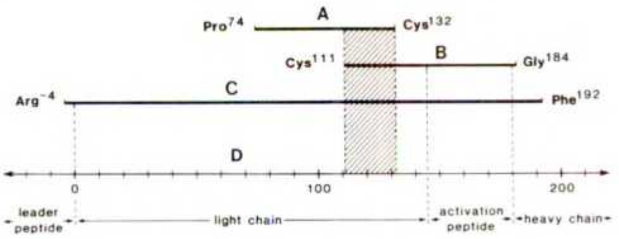

Fig 2. Factor IX polypeptide regions encoded by three immunoreactive clones: (A) Gor9; Pro^{74}-Cys^{132}; (B) Gor1; Cys^{111}-Gly^{184} (C); Gor 7; Arg^{-4}-Phe^{192}; (D) factor IX. The antigenic site recognized by IX-30 must lie within the overlapping region (shaded).

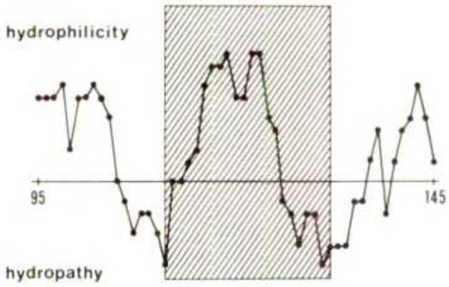

Fig 3. Hydrophilicity/hydropathy profile. The profile is given for residues 95 through 145 of zymogen factor IX. The region mapped with IX-30 is shaded.

The immunoreactivity of IX-30 was further assessed by immunoblotting. Lanes 1 through 4 in Fig 5 show protein (Coomassie blue) staining of the factor IX, factor IXa, factor X, and protein C preparations. Lanes 5 through 8 show corresponding results obtained by immunological staining with IX-30. The factor IX (lane 1) appears to migrate as a single prominent band, as do factor X (lane 3) and protein C (lane 4). The two prominent bands in lane 2 (factor IXa) are due to incomplete activation of the zymogen.

The immunological staining with IX-30 reveals additional bands in the factor IX and IXa preparations (lanes 5 and 6). These bands correspond to specific degradation products lacking a carboxy-terminal segment of the heavy chain.[16] Although barely visible by protein staining, their prominence in the immunoblot is due to the extreme sensitivity of detection. No immunoreactivity was detected in the protein C preparation (lane 8). The factor X preparation contained a small amount of immunoreactive material (lane 7) with an electrophoretic mobility matching that of factor IX. We now interpret this to mean that there is a trace factor IX contaminant in the factor X preparation rather than a limited cross-reactivity of IX-30 with factor X as suggested previously.[17] No immunoreactivity was observed to correspond to the major band of factor X protein. Reduced factor IX and IXa were also reactive in immunoblots. A low molecular weight band tentatively identified as the light

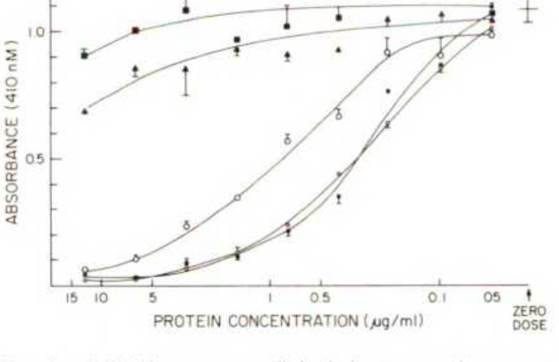

Fig 4. Inhibition enzyme-linked immunosorbent assays (ELISA). Large filled squares, human protein C; triangles, human factor X; large open circles, human factor IX; small open circles, human factor IXa; small filled squares, bovine factor IX.

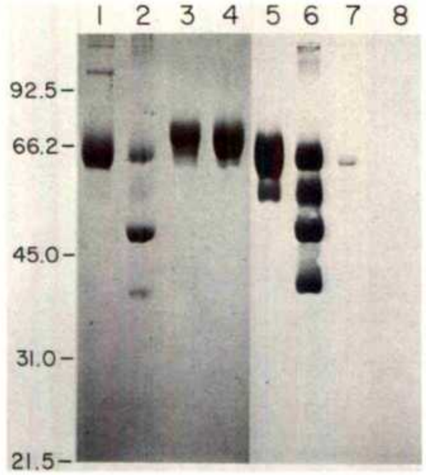

Fig 5. Immunoblotting. Lanes 1 through 4 show the Coomassie blue staining of human factor IX, human factor IXa, human factor X, and human protein C, respectively. Lanes 5 through 8 show the immunological staining with IX-30 of a similar gel.

chain of factor IXa was detected in the activated sample (data not shown).

DISCUSSION

IX-30 epitope. We used the recombinant DNA approach first described by Nunberg and co-workers[5] to map an antigenic site in human factor IX. We showed that the epitope recognized by monoclonal antibody IX-30 lies within a region of 22 amino acid residues in the light chain, extending from Cys^{111} to Cys^{132}. These results were obtained with the most potent of the nine tested monoclonal antbodies. (IX-30 has an ELISA titer of 1×10^6 even in the absence of calcium ions). It may be simply for this reason that only IX-30 gave positive results, but rescreening with the other monoclonals at lesser dilution yielded no additional clones.

The ability of IX-30 to recognize factor IX and factor IXa in immunoblots on a charged solid-phase matrix suggests that the epitope is primarily sequential in nature. It also is clear from the results presented here that the antigenic site detected by IX-30 can be recognized in various peptide contexts. The peptide sequence mapped with IX-30 represents a region of local hydrophilicity in the factor IX protein, consistent with the analysis of Hopp and Woods, which predicts that such sites will frequently act as antigenic determinants.

Although IX-30 reacts only with factor IX, the region mapped with this antibody has interesting homologies with several other proteins, as has been noted previously by others[18,19] (Table 1). There is a homology with a consensus sequence for a group of proteins that includes not only the clotting factors, but several growth factors, the LDL receptor protein, and possibly a *Vaccinia* viral protein. The presence of sites analogous to the IX-30 epitope in other proteins could be taken as evidence of a conserved structural role for this domain. If the region has functional significance, one might imagine that it is involved with a cell-surface receptor. A recent report of a coagulation pathway dependent on factor IX binding to perturbed endothelial cells may lend support to this hypothesis.[20] However, any such evidence will have to be reconciled with the observation that IX-30 does not interfere with coagulation in vitro, even at high concentrations (data not shown).

Generality of the method. Further studies will obviously be required to determine the overall usefulness of this method. Several factors are important for its success. On a purely random basis, only one in six recombinants will be expected to generate a factor IX-specific peptide. This is because an inserted fragment must have both the appropriate orientation (one of two possibilities) and the correct reading frame (one of three) in order to be expressed. In practice, this is not a severe limitation, however, since it is possible to generate and screen very large populations of recombinants.

A more important consideration is that the experimental system requires expression of the antigen as part of a "fusion protein" consisting primarily of bacterial β-galactosidase. Many antigenic sites on the original immunizing protein may not be regenerated in this context. Some epitopes may involve residues that are juxtaposed in the folded protein but distant in terms of the primary amino acid (or DNA) sequence. Others may involve chemical moieties of post-translational origin. These could include any of the carbohydrate additions, β-hydroxylation at Asp^{64},[21] or the well-documented γ-carboxylation of glutamic acid residues in the amino-terminal region.[22]

It may be, therefore, that only a small percentage of monoclonal antibodies raised against the factor IX protein will recognize short "primary-sequence" epitopes such as the one described in this report. However, when one already has a large number of monoclonals, it will probably be worthwhile to attempt this approach. The primary screening can be done with a mixture of many monoclonals, and useful information will be obtained even if only a few yield positive clones. As an alternative means to the same end, one might choose to generate region-specific probes by raising antisera against synthetic peptides.[23]

Table 1. Homologies With Other Proteins

Region	111												124									132
a	C	T	E	G	Y	R	L	A	E	N	Q	K	S	C	E	P	A	V	P	F	P	C
b	C	T	D	G	Y	R	L	A	E	D	Q	K	S	C	E	P	A	V	P	F	P	C
c	C	A	R	G	Y	T	L	A	D	N	G	K	A	C	I	P	T	G	P	Y	P	C
d	C	A	P	G	Y	K	L	G	D	D	L	L	Q	C	H	P	A	V	K	F	P	C
e	C	X	X	G	Y/F	X	L	X	X	D	X	K	X	C								

Regions corresponding to the IX-30 epitope are given in single-letter amino acid code: (a) Human factor IX;[9] Cys^{111}-Cys^{132}. (b) Bovine factor IX;[24] Cys^{111}-Cys^{132}. (c) Human factor X;[25] Cys^{111}-Cys^{132}. (d) Human protein C;[26] Cys^{120}-Cys^{141}. (e) Consensus sequence for homologous regions in various other proteins.[18,19] Residues matching the consensus are underlined.

REFERENCES

1. McGraw RA, Davis LM, Lundblad RL, Stafford DW, Roberts HR: Structure and function of factor IX: Defects in hemophilia B, in Ruggeri Z (ed): Clinics in Haematology. Coagulation Disorders, vol 14. London, Saunders, 1985, p 359

2. Young RA, Davis RW: Efficient isolation of genes by using antibody probes. Proc Natl Acad Sci USA 80:1194, 1983

3. Schwarzbauer JE, Paul JI, Hynes RO: On the origin of species of fibronectin. Proc Natl Acad Sci USA 82:1424, 1985

4. Nunberg JH, Rodgers G, Gilbert JH, Snead RM: Method to map antigenic determinants recognized by monoclonal antibodies: Localization of a determinant of virus neutralization on the feline leukemia virus envelope protein gp70. Proc Natl Acad Sci USA 81:3675, 1984

5. Smith GJ, McFarland RD, Reisner HM, Hudson GS: Lymphoblastoid cell-produced immunoglobulins. Preparative purification from culture medium by hydroxylapatite chromatography. Anal Biochem 141:432, 1984

6. Bradley LA, Franco EL, Reisner HM: The use of monoclonal antibodies in an enzyme-immunoassay for factor VIII-related antigen. Clin Chem 30:87, 1984

7. Laemmli UK: Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

8. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. New York, Cold Spring Harbor Laboratory, 1982

9. McGraw RA, Davis LM, Noyes CM, Lundblad RL, Roberts HR, Graham JB, Stafford DW: Evidence for a prevalent dimorphism in the activation peptide of human coagulation factor IX. Proc Natl Acad Sci USA 82:2847, 1985

10. Grosveld FG, Dahl HHM, DeBoer E, Flavell RA: Isolation of beta globin genes from a human cosmid library. Gene 13:227, 1981

11. Sanger F, Nicklen S, Coulsen AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463, 1977

12. Messing J, Crea R, Seeburg PH: A system for shotgun DNA sequencing. Nucleic Acids Res 9:309, 1981

13. McGraw RA: Dideoxy DNA sequencing with end-labeled oligonucleotide primers. Anal Biochem 143:298, 1984

14. Kyte J, Doolittle RF: A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105, 1982

15. Hopp TP, Woods KR: Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78:3824, 1981

16. Kisiel W, Smith KJ, McMullen BA: Proteolytic inactivation of blood coagulation factor IX by alpha-thrombin. Thromb Haemost 54:274, 1985 (abstr)

17. McGraw RA, Frazier LD, De Serres M, Stafford D, Reisner HM: Antigenic determinants in human coagulation factor IX—Immunological screening and DNA sequence analysis of recombinant phage map a monoclonal antibody to residues 111–132. Thromb Haemost 54:274, 1985 (abstr)

18. Blomquist MC, Hunt LT, and Barker WC: *Vaccinia* virus 19-kilodalton protein: Relationship to several mammalian proteins, including two growth factors. Proc Natl Acad Sci USA 81:7363, 1984

19. Sudhof TC, Goldstein JL, Brown MS, Russell DW: The LDL receptor gene: A mosaic of exons shared with different proteins. Science 228:815, 1985

20. Stern D, Nawroth P, Handley D, Kisiel W: An endothelial cell-dependent pathway of coagulation. Proc Natl Acad Sci USA 82:2523, 1985

21. McMullen BA, Fujikawa K, Kisiel W: The occurrence of beta-hydroxyaspartic acid in the vitamin K-dependent blood coagulation zymogens. Biochem Biophys Res Commun 115:8, 1983

22. Fryklund L, Borg H, Anderson L-O: Amino terminal sequence of human factor IX: Presence of gamma-carboxy-glutamyl residues. FEBS Lett 65:187, 1976

23. Sutcliffe JG, Shinnick TM, Green N, Lerner RA: Antibodies that react with predetermined sites on proteins. Science 219:660, 1983

24. Katayama K, Ericsson LH, Enfield DL, Walsh KA, Neurath H, Davie EW, Titani K: Comparison of amino acid sequence of bovine coagulation factor IX (Christmas factor) with that of other vitamin K-dependent plasma proteins. Proc Natl Acad Sci USA 76:4990, 1979

25. Fung MR, Hay CW, MacGillivray RTA: Characterization of an almost full-length cDNA coding for human blood coagulation factor X. Proc Natl Acad Sci USA 82:3591, 1985

26. Foster DC, Yoshitake S, Davie EW: The nucleotide sequence of the gene for human protein C. Proc Natl Acad Sci USA 82:4673, 1985

Analysis of Natural Killer Cells in Patients With Aplastic Anemia

By Pedro Gascón, Nicholas Zoumbos, and Neal Young

We have analyzed natural killer (NK) cells in 43 patients with severe aplastic anemia, using cytotoxicity assays and microfluorometry with monoclonal antibodies, prior to and after treatment with antithymocyte globulin (ATG). Before treatment, natural killer cell activity (NK$_a$) in both peripheral blood and bone marrow was markedly decreased in 76% of patients as compared with normal controls. Although we have measured low NK$_a$ in patients receiving large numbers of blood transfusions ($\bar{x}$ = 150 U of RBCs), six aplastic patients had low NK$_a$ in the absence of transfusions, and the average number of transfusions in the total population was low ($\bar{x}$ = 24). Purification of larger granular lymphocytes (LGLs) from peripheral blood of aplastic anemia patients failed to recover significant NK$_a$. Most of these large granular lymphocytes showed few azurophilic granules. NK$_a$ was appropriately enhanced in these patients' samples by exposure of mononuclear cells to either interleukin 2 (IL-2) or interferon (IFN). Analysis of peripheral blood phenotypic markers showed that cells bearing Leu 7 antigen were in the normal range in aplastic anemia ($\bar{x}$ = 12% ± 2%; normal = 16% ± 2%), but there was a deficiency of Leu 11$^+$ cells ($\bar{x}$ = 8% ± 2%; normal = 15% ± 2%). The number of Leu 11$^+$ cells was well correlated with NK$_a$. In 13 of 22 patients treated with ATG, NK$_a$ returned to the normal range, and recovery of NK$_a$ was correlated to hematopoietic recovery. Our results suggest that deficient NK$_a$ is an intrinsic feature of aplastic anemia, and that the circulating cells in this disease are of the pre-NK cell stage.

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NATURAL KILLER (NK) cells are defined by their ability to lyse spontaneously various tumor cells, cells infected with virus, and some normal cells.[1,2] In humans, NK cells have been identified as large granular lymphocytes (LGLs),[3,4] and they bear the cell surface antigen markers Leu 7 and Leu 11.[5-7] The lineage of NK cells is controversial, as they display qualities of both T lymphocytes and myelomonocytes.[8-11] Animal studies have implicated these cells in immune surveillance of transformed malignant and virus-infected cells.[1,12] Clinical studies of NK cells have shown low activity in various diseases, including systemic lupus erythematosus,[13] chronic lymphocytic leukemia,[14] Chediak-Higashi syndrome,[15] X-linked lymphoproliferative syndrome,[16] and others; therefore, their functional significance in humans remains uncertain.

NK cells have been implicated in the normal regulation of hematopoiesis.[17] Murine NK cells recognize normal bone marrow cells, as measured in competition experiments using a tumor target cell line,[18] and NK cells can inhibit murine stem cell (CFU-S) proliferation.[19] NK cells inhibit myeloid, or CFU-C–derived,[20] and erythroid, or CFU-E–derived,[21] colony formation by human bone marrow. Furthermore, NK cells may play an important role in the rejection of transplanted bone marrow cells.[22] The ability of NK cells to inhibit hematopoietic cell proliferation in vitro has led to their implication in the pathogenesis of bone marrow failure states.[20]

Several lines of evidence have suggested that aplastic anemia may be immunologically mediated.[23] Important soluble regulators of the immune system are abnormal in aplastic anemia, including low interleukin 1 (IL-1) production by monocytes (P. Gascon et al, manuscript in preparation) and high IL-2[24] and γ-interferon (γ-IFN) production in vitro.[25] Because these monokines and lymphokines are both produced by NK cells and in turn are capable of regulating their function, we undertook a study of NK cells in a large number of patients with aplastic anemia.

MATERIALS AND METHODS

Study Population

Forty-three patients (mean age 34 ± 16 years) with severe aplastic anemia, as defined by peripheral blood counts and bone marrow biopsy,[26] were studied. Twenty-three patients had acute disease (less than three months from the time of diagnosis to study), and 20 had chronic disease. All patients were studied at least once for natural cytotoxic activity, and assays in ten patients were repeated twice prior to antithymocyte globulin (ATG) treatment. Tests were carried out at admission and at 30, 60, 90, and 180 days after completion of treatment. Thirty-seven patients had received some platelet or erythrocyte transfusions prior to the study. None of the patients showed clinical evidence of active viral infection, and their titers of antibodies to Epstein-Barr virus, cytomegalovirus, and hepatitis A and B virus were lower than were those of multiply transfused patients.[27] Sixty-four normal laboratory personnel (mean age 31 ± 8 years) of three races served as controls.

Preparation of Cells

Peripheral blood lymphocytes. Fresh blood was drawn into syringes, containing 10 U/mL of preservative-free heparin (O'Neill and Feldman, St Louis), and mononuclear cells were separated on Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md) gradients. Cells were washed three times and resuspended in RPMI 1640 medium (M. A. Bioproducts, Walkersville, Md) supplemented with 2 mmol/L of glutamine, 100 U/mL penicillin/streptomycin (M. A. Bioproducts), and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, lot 100378, Sterile Systems, Inc, Logan, Utah).

Lymphocytes were purified by two-step adherence–nonadherence separation, on plastic surfaces and nylon wool columns, and sedimentation on Percoll gradients. Low-density cells, which are mostly LGLs, and high-density cells (predominantly T lymphocytes) were obtained by centrifugation on a discontinuous gradient of Percoll (Pharmacia, Uppsala, Sweden).[28] An essential step before the gradient centrifugation was the rigorous removal of adherent cells that tended to band in the fractions close to those of LGLs. Almost complete removal of adherent cells was achieved by incubation on plastic Petri dishes of mononuclear cells obtained after Ficoll-Hypaque centrifugation (3003; Falcon Labware, Division of Becton Dickinson, Oxnard, Calif) for one hour at 37 °C and a further

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incubation on nylon columns (Associated Biomedic Systems, Inc, Buffalo) for 30 minutes at 37 °C. Nonadherent cells were applied to a seven-layer discontinuous Percoll density gradient that varied in concentration from 2.5% to 40%–55%. Cells from all layers were examined by morphology using Giemsa-stained cytopreps.[28] LGLs were recovered from fractions II and III at 42.5% to 45% Percoll.

Bone marrow lymphocytes. Bone marrow was aspirated into 1 mL of Iscove's modification of Dulbecco's medium (IMDM) and 250 U of preservative-free heparin. Bone marrow mononuclear cells were obtained by the same procedure as is used to obtain peripheral blood. Informed consent was obtained from patients and normal subjects according to protocols approved by the NHLBI review board.

Flow Cytometry

T lymphocyte subpopulations from peripheral blood mononuclear cells and from Percoll fractions I, II, III, and IV were quantitated by direct immunofluorescence using either fluorescein-conjugated or phycoerythrin-conjugated monoclonal antibodies Leu 1, Leu 2, Leu 3, Leu 7, Leu 11a, and anti-HLA-DR (nonpolymorphic, clone L243) (Becton Dickinson, Sunnyvale, Calif); mouse-IgG conjugated to the appropriate fluorescent molecule served as a negative control. T lymphocytes were suspended in Hank's balanced salt solution (HBSS) without phenol red (M. A. Bioproducts), containing 0.1% sodium azide and 0.1% bovine serum albumin (BSA). After centrifugation, 1×10^6 cells were incubated with 5 μL of appropriate monoclonal reagent at 4 °C for 30 minutes and then washed twice. When double labeling was used, a second monoclonal antibody conjugated either with fluorescein or with phycoerythrin was added for 30 minutes at 4 °C; the cells were then washed twice. Flow microfluorometry was performed using a FACS II (Becton Dickinson FACS System); data were selected, stored, and analyzed using a PD 11/35 computer (Digital Equipment Corp, Marlboro, Mass).[29] Cell sorting was performed in some experiments using the NK cell markers Leu 7 and Leu 11a. Morphology of 200 sorted cells was then examined, using Giemsa-stained cytopreps,[28] by one of the authors and an independent hematologist.

NK Cell Assay

Natural killer cell activity (NK_a) was assayed by release of ^{51}chromium (^{51}Cr) from K562 target cells.[27] Target cells were labeled with 200 μCi of ^{51}Cr (Amersham, Arlington Heights, Ill) for one hour at 37 °C and were washed extensively before use. Target cells, 5×10^3 ^{51}Cr-labeled K562 in 100 μL of medium, were dispensed into 96-well, round-bottom microtiter plates (Costar, Cambridge, Mass). Mononuclear effector cells (100 μL) were added to wells in triplicate at serial effector-target cell ratios ranging from 100:1 to 6.25:1. One hundred microliters of supernatant was harvested for each well, and the radioactivity was measured in a gamma counter (Beckman 4000; Beckman Instruments, Inc, Irvine, Calif). Duplicate experiments were carried out for harvesting of the supernatants at 4 and 18 hours. Spontaneous release of ^{51}Cr was always $<10\%$ of total release for four hours and $<30\%$ for 18 hours. Total release was determined by addition of 100 μL of 5% Triton X-100 (Research Products International Corp, Elk Grove Village, Ill). The percentage of cytotoxicity was calculated according to the following formula: 100 × cpm (experimental release) − cpm (spontaneous release)/cpm (total release) − cpm (spontaneous release).

Effect of IL-2 and IFN on NK_a. IL-2 and IFN, two known boosting agents of NK activity,[30,31] were used in our experiments. Final concentration of 200 U/mL of highly purified α-IFN (New York Blood Center) and 30 U/mL of monoclonal antibody affinity-purified IL-2 obtained from the Jurkat cell line (Dupont Laboratories, Glenolden, Pa), provided by Dr Julie Djeu, were added to different wells containing effector cells. After one hour of incubation at 37 °C in a 5% CO_2 humidifier chamber, labeled target cells were added to the respective wells. Supernatants were harvested four hours later and assayed as described above.

Statistical analysis. Data were analyzed using Student's *t* test, the two-tailed nonparametric Wilcoxon test, the Fisher exact two-tailed test, and linear regression analysis.

RESULTS

NK_a in peripheral blood. NK_a in the blood of most patients with aplastic anemia was markedly reduced in comparison to that of normal individuals ($P < .002$). Titrations at different effector-target ratios in the first 38 patients studied are shown in Fig 1; of these 38 patients, 18 had no detectable NK_a; 11 other patients had activity <2 SD from the normal mean. Cytotoxicity for K562 leukemic cell targets increased with time, ie, from 4 to 18 hours of incubation, but did not reach the level of normal controls at any given time at effector-target ratios ranging from 100:1 to 6.25:1. NK_a in acute aplastic anemia patients (n = 18; $\bar{x} \pm SE = 11 \pm 3$) was not statistically different from chronic patients (n = 20; $\bar{x} \pm SE = 19 \pm 4$). To determine if decreased NK_a was due to the presence of a soluble factor in the circulation of patients with aplastic anemia, sera from six patients was preincubated for one hour at 37 °C with normal peripheral blood mononuclear cells (PBMNCs) before addition of target cells; under these circumstances, NK_a of the normal cells was unaffected (data not shown).

NK_a in bone marrow. Because NK cells have been suggested as possibly mediating hematopoietic suppression in aplastic anemia,[21,32] we measured NK_a in the bone marrow of 12 patients. Measurement of a low proportion of total and helper T cells in the bone marrow as compared with the peripheral blood suggested that dilution of the marrow samples by peripheral blood was minimal.[33] As shown in Fig 2, mean NK_a in aplastic bone marrow was decreased as compared with normal and to approximately the same degree

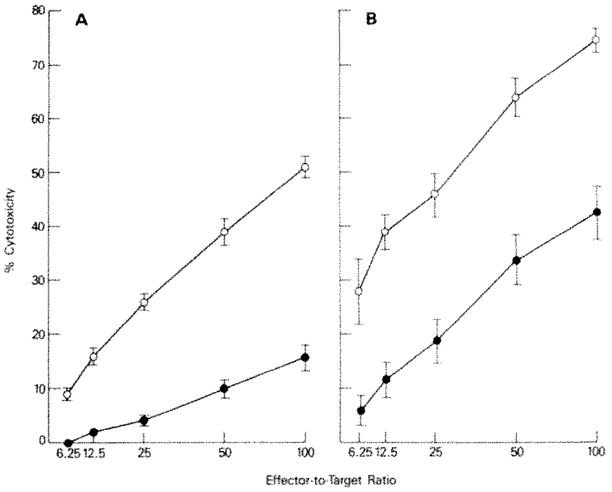

Fig 1. Peripheral blood natural killer cell activity (NK_a) in aplastic anemia patients, n = 38 (o) as compared with normal controls, n = 60 (o): (A) at four hours ($P < .0001$) of incubation; (B) at 18 hours of incubation ($P < .005$). Bars represent SE.

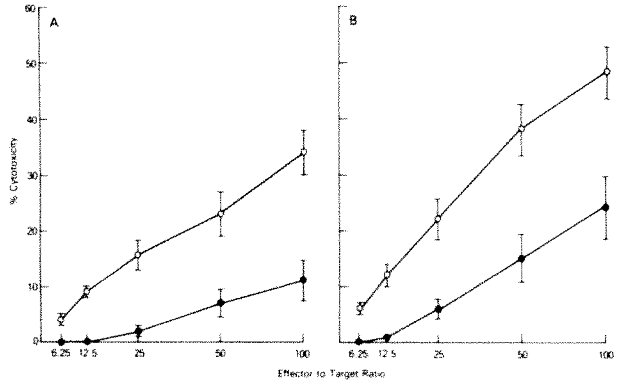

Fig 2. Bone marrow natural killer cell activity (NK_a) in aplastic anemia patients, n = 12 (o) as compared with normal controls, n = 12 (o): (A) at four hours ($P < .01$) of incubation; (B) at 18 hours of incubation ($P < .001$). Bars represent SE.

as was observed in blood ($P < .001$). Eight of the 12 aplastic bone marrows assayed showed NK_a below 2 SD from the normal mean. These results indicated that high NK_a was not concentrated in the target organ of aplastic anemia.

Effect of lymphokines on NK_a. To determine whether lymphokines such as IFN and IL-2 could activate NK_a in aplastic anemia patients to the level of normal controls, either IFN, IL-2, or medium was added to PBMNCs or bone marrow mononuclear cells of the patients and normal individuals for one hour at 37 °C before they were assayed for NK function. These lymphokines increased NK_a in both PBMNCs and bone marrow mononuclear cells from aplastic anemia patients as efficiently as in those of normal subjects (Table 1).

Purification of LGLs. NK_a has been associated with LGLs that can be isolated by discontinuous gradient centrifugation between 42.5% to 45% Percoll (fractions II and III) centrifugation. To determine whether, as a result of the pancytopenic process, the number of NK cells was low but that those that were present were functionally normal, LGLs were isolated from PBMNC preparations of 13 patients with aplastic anemia and enumerated before NK_a was assayed. No activity was recovered from fractions II and III in seven cases, and only minimal activity was seen in the rest. Although the same number of LGLs were tested for activity as in normal controls, the LGLs in aplastic anemia had defective NK_a. In only one case, in which NK_a in unfractionated cells was in the low normal range, was normal activity recovered following Percoll gradient centrifugation.

LGL morphology. The light-microscopy morphology of LGLs from patients with aplastic anemia appeared abnormal (Fig 3) in that they showed a marked lack of granules in most of the LGLs in comparison with normal LGLs. In preparations from normal individuals, > 90% of LGLs showed prominent azurophilic granules, and the remaining cells showed fine granulation. In contrast, in preparations from aplastic anemia patients, > 20% of LGLs lacked granules, > 40% had fine granulation, and the remaining cells showed only a few granules. These data suggest that NK cells were qualitatively as well as numerically defective in aplastic anemia.

Analysis of phenotypic markers. Two monoclonal antibodies, termed Leu 7 and Leu 11, have been described to bind with some degree of specificity to NK cells,[4,6] with Leu 11-bearing cells being the more effective NK cells in functional assays.[7] To assess the NK cell phenotypes that were present in aplastic anemia, flow microfluorometry of surface antigens was performed on PBMNCs of 29 patients with aplastic anemia and 19 normal controls. The mean proportion of cells bearing the Leu 7 antigen in the patient population was not significantly different from normal (Table 2), although 30% of patients had much higher levels of Leu 7^+ cells than did normal subjects. There was no positive correlation between the number of Leu 7^+ cells and NK_a in aplastic anemia patients ($r = .1$) (Fig 4) such as was found for the normal controls ($r = .4$). In contrast, the number of Leu 11-bearing cells in aplastic anemia correlated to NK_a ($r = .64$) as it did among normals ($r = .52$) (Fig 4). The absolute numbers of both Leu 7^+ and Leu 11^+ cells in the circulation of patients with aplastic anemia were markedly decreased ($P < .05$).

In three patients, cells from Percoll gradient layers I, II, III, and IV were analyzed for NK cell markers (Table 3). There was low NK_a in all fractions, which correlated with the low proportion of Leu 11^+ cells, analogous to the results in peripheral blood.

Table 1. Effect of Lymphokine Stimulation on NK_a in Aplastic Anemia

		Percentage of NK			
		4-h Incubation			18-h Incubation
Source of Effector Cells		Medium 100:1*	+IL-2 100:1	+IFN 100:1	Medium 100:1
Peripheral blood	NC	51 ± 1 (60)	61 ± 4 (15)	67 ± 4 (15)	71 ± 3 (25)
	AA	15 ± 2 (38)	42 ± 5 (15)	39 ± 6 (16)	43 ± 4 (27)
	P†	<.0001	<.02	<.002	<.0001
Bone marrow	NC	32 ± 4 (12)	49 ± 5 (8)	54 ± 5 (8)	49 ± 2 (10)
	AA	14 ± 2 (16)	24 ± 5 (12)	23 ± 5 (12)	26 ± 5 (12)
	P†	<.002	<.01	<.001	<.01

NK_a, natural killer cell activity; NC, normal controls; AA, aplastic anemia patients.

Results are given as mean ± SE. Number of patients studied in parentheses.

*Effector-target ratio.

†Two-tailed *P* value using the nonparametric Wilcoxon test.

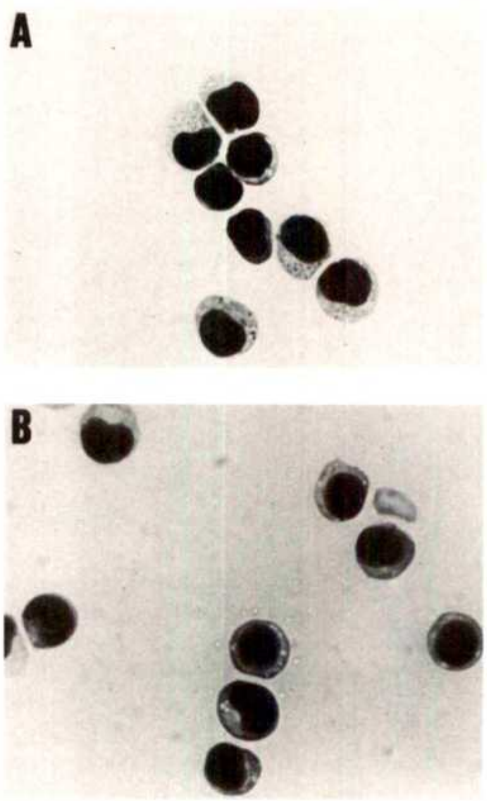

Fig 3. Wright's Giemsa cytospin preparation of stained cells from fractions II and III of Percoll gradients. (A) Large granular lymphocytes (LGLs) from a normal control; (B) LGLs from an aplastic anemia patient. Both cell preparations were assayed simultaneously. Aplastic anemia LGLs showed few azurophilic cytoplasmic granulations. One experiment representative of eight is shown.

Table 2. Phenotype Markers for NK in PBMNCs of Aplastic Anemia Patients

Marker	Pre-ATG	Post-ATG	Normal Controls
Leu-7 (%)	12 ± 2 (29)	21 ± 7 (10)*	16 ± 2 (19)
Leu-11 (%)	8 ± 2 (20)	12 ± 6 (10)	15 ± 2 (10)
DR Leu-3/DR Leu-2 (%)	0.9 ± 0.2 (15)	0.8 ± 0.1 (10)	4.2 ± 4 (10)

NK, natural killer cells; PBMNCs, peripheral blood mononuclear cells; ATG, antithymocyte globulin.

Results are given as mean ± SE. Number of patients studied in parentheses; %, percentage of total peripheral blood lymphocytes positive.

*Three responders and seven nonresponders, one studied 1 month and nine studied 3 months after ATG therapy.

In normal individuals, cells bearing Leu 11 outnumber those bearing Leu 7 ($\bar{x}$ ratio = 1.35 ± 0.28). However, in the aplastic population, Leu 7 cells predominated ($\bar{x}$ ratio = 0.85 ± 0.53) and ratios of 11 of 21 patients were below 2 SD from the normal mean. In addition, we also assessed the phenotypes of T cells in PBMNCs of aplastic anemia patients. NK_a was directly correlated ($r = .62$) to the relative proportion of T helper and T suppressor cells (Leu 3/Leu 2) (data not shown). NK_a had a somewhat better correlation to the activated helper-suppressor lymphocyte ratio ($r = .79$), which was defined by two-color fluorometry using anti-HLA-DR monoclonal antibody as a marker of T cell activation (DR^+Leu 3/DR^+Leu 2) (Fig 4). A similar relationship existed for normal controls (n = 10), in whom the helper-suppressor lymphocyte ratio highly correlated with NK_a ($r = .86$) as did the activated helper-suppressor lymphocyte ratio ($r = .98$).

Effect of therapy on NK_a. Between 40% and 50% of patients with aplastic anemia showed a hematopoietic response to therapy with ATG.[31] To determine the effects of ATG on recovery of NK_a, we prospectively studied NK_a in 22 patients prior to and following ATG therapy (Fig 5). In this series, 13 patients became transfusion-independent. NK_a increased into the normal range in a total of 13 patients; in four patients NK_a decreased following therapy, and in five patients it remained unchanged. There was a strong correlation between hematopoietic recovery and return to normal of NK_a: 11 of 13 patients who responded hematopoietically recovered normal NK_a ($P < .01$ by the Fisher exact test) (Table 4). However, in one patient who had a return to normal blood counts, NK_a remained absent. NK_a increased in two of nine patients who failed to respond hematologically to ATG treatment.

DISCUSSION

NK_a in the blood of patients with aplastic anemia was markedly reduced in the majority of the 38 patients we now report and in a previously published series.[32] We also provide

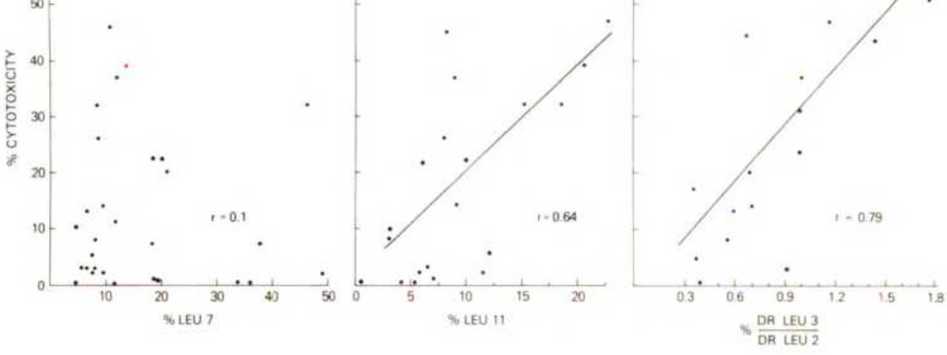

Fig 4. Natural killer (NK) cell phenotypic markers in peripheral blood mononuclear cells from aplastic anemia patients: (A) Leu-7 plotted against NK_a ($r = .1$); (B) Leu 11 correlated with NK_a ($r = .64$); (C) correlation between the activated helper-suppressor lymphocyte ratio and NK_a ($r = .79$). NK_a is expressed as percentage of cytotoxicity at an effector-target ratio of 100:1.

Table 3. NK_a and Surface Phenotype of Percoll Gradient Fractions

Marker		Fraction No.		
		I	II + III	IV
NK_a (%)	AA	0	12 ± 9	5 ± 5
	NC	10 ± 2	78 ± 10	30 ± 4
Leu-7 (%)	AA	20 ± 4	48 ± 6	26 ± 11
	NC	14 ± 2	44 ± 10	19 ± 8
Leu-11 (%)	AA	4 ± 2	18 ± 8	8 ± 3
	NC	18 ± 4	67 ± 6	23 ± 2
Leu 1 (%)	AA	ND	22 ± 3	92 ± 7
	NC	ND	14 ± 6	88 ± 6

AA, aplastic anemia (n = 3); baseline natural killer cell activity for AA (NK_a) = 8% ± 12%. NC, normal controls (n = 3); baseline NK_a for NC = 42% ± 11%. ND, not done (insufficient cells for analysis by flow cytometry). Results are expressed as ($\bar{X}$ ± SEM).

Table 4. Effect of Post-ATG-NK_a v Hematopoietic Recovery

		NK_a	
		Increased*	No Change or Decreased
HR	+	11	2
	−	2	7

Natural killer cell activity (NK_a) was determined one or three months after beginning of treatment and compared with values obtained before therapy. The results were highly significant by the Fisher two-tail test ($P < .01$). HR, hematopoietic response.

*Defined as post-ATG value > 30% higher than pre-ATG value.

new evidence that correspondingly low NK_a also exists in bone marrow mononuclear cells obtained from patients with aplastic anemia. These results suggest that defective NK function is a consequence of the underlying bone marrow failure and therefore do not support the suggestion[21,32] that hematopoietic suppression in aplastic anemia is mediated by NK cells, at least as these cells are functionally defined. Abnormal IFN production[25] and activated suppressor lymphocytes[34] have both been implicated in immune-mediated hematopoietic suppression. We cannot rigorously exclude the possibility that NK cells play a role in this suppression by contributing to IFN production or as members of a suppressor lymphocyte population, or that decreased NK cells by phenotype and activity are the result of high activity of such cells at an earlier stage of the disease.

Low NK_a could be the result of a quantitative or qualitative defect, either a deficiency in the number of NK cells produced or the ability of NK cells to function as measured in the K562 cell cytotoxicity assay. The pancytopenia that characterizes aplastic anemia probably encompasses more than erythrocytes, platelets, and granulocytes. For example, monocytes,[35] B lymphocytes,[36] and helper/inducer lymphocytes[33] also have been reported to be decreased in these patients. NK cells have been assigned to both monocytic[37] and lymphocytic[38] lineages. We were unable to recover normal numbers of LGLs by density gradient fractionation of mononuclear cells, suggesting that NK cells in fact are decreased in number in aplastic anemia. The very low absolute numbers of Leu 7 and Leu 11 antigen-bearing mononuclear cells in patients with aplastic anemia indicates that NK cell numbers are reduced as a function of the pancytopenia that characterizes aplastic anemia. In addition to the reduced number of NK phenotypes, the NK cells with LGL morphology showed defective cytotoxic function. The recovery of hematopoietic function in treated aplastic anemia patients generally was associated with an increase in NK_a and NK cell phenotype.

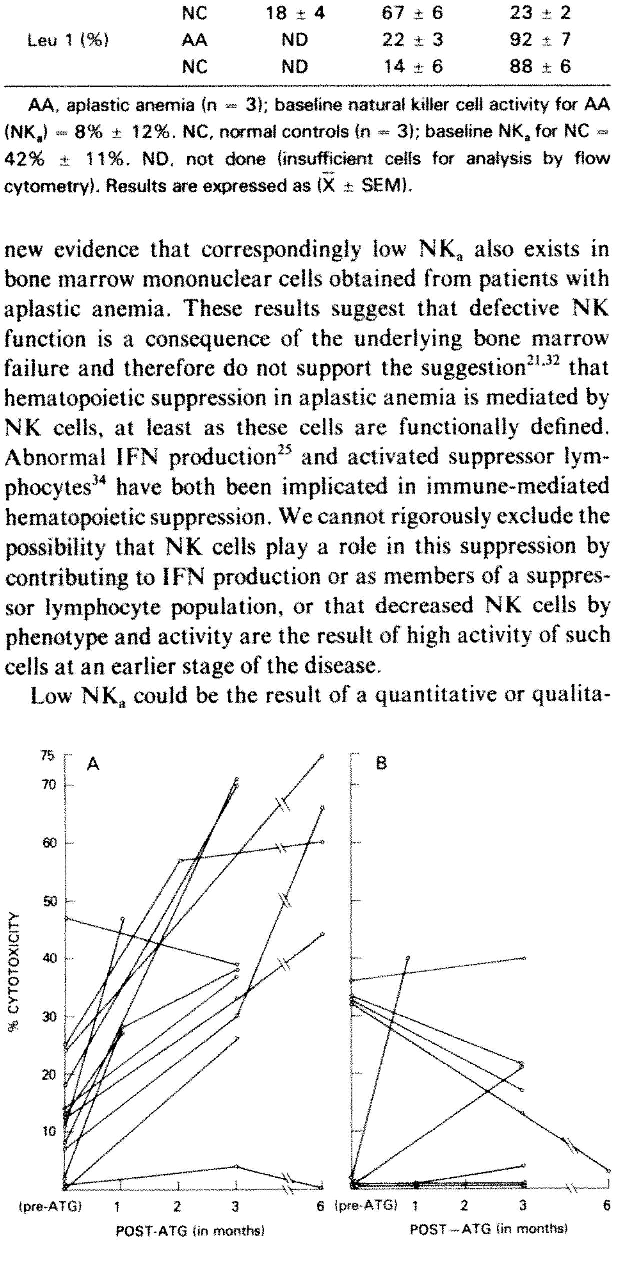

Fig 5. The effect of antithymocyte globulin (ATG) therapy on natural killer cell activity (NK_a) with (n = 13) (A) and without (n = 9) (B) hematologic recovery. NK_a is expressed as percentage of cytotoxicity at an effector-target ratio of 100:1. Blood counts at three months after ATG therapy showed: Absolute reticulocytes ($\times 10^4/\mu L$) for A were 5.5 ± 3; for B, they were 2.6 ± 1.2 (with transfusion support). Absolute neutrophils (—/μL) for A were 1211 ± 901, and for B were 742 ± 700. Platelets ($\times 10^3/\mu L$) for A were 43 ± 52, and for B were 9 ± 4.

In spite of impairment in spontaneous NK_a, aplastic anemia patients have lymphokine-responsive NK cells in both peripheral blood and bone marrow. These cells readily responded to activation by IFN or IL-2 in vitro, and NK_a could be reconstituted to the normal spontaneous range by either lymphokine. Therefore, lymphokine-responsive NK cells are intact in aplastic anemia, although there is a deficiency of cells with spontaneous NK_a.

Phenotypic analysis of residual NK cells in the peripheral blood of aplastic anemia patients showed that there was a relative predominance of Leu 7^+ cells and a marked reduction in the number of Leu 11^+ cells. Leu 11^+, Leu 7^- NK cells have been characterized as having the highest spontaneous NK_a.[7] Our data suggest that most cells bearing the NK phenotype in aplastic anemia may be characterized instead as lymphokine-responsive NK precursor cells with low spontaneous NK_a. The small number of granules in LGLs isolated from aplastic anemia patients also indicates that most of the cells are in an inactive form, possibly representing the precytotoxic stage.[39,40] The results are all consistent with an immature but lymphokine-responsive population of NK cells contributing to the low NK_a measured in blood and bone marrow.

How can low NK_a be related to the abnormalities of lymphokine production in aplastic anemia?[24,25] IFN and IL-2 stimulate NK_a, and the production of these two lymphokines by PBMNCs in vitro is very high;[24,25] serum levels of IFN are also elevated in 30% of patients with aplastic anemia and in the bone marrow of almost all cases.[24] One explanation for this possible discordance is the differing effect of short-term and long-term exposure to IFN in vivo. Infusion of IFN in cancer patients increases NK_a during the first 24 hours of administration, but thereafter NK_a is low.[41] In addition, acquired immunodeficiency syndrome (AIDS) patients who reportedly have elevated circulating IFN levels[42] are defective in NK function,[43] similar to aplastic anemia patients. The consistent association of high IFN levels and low NK_a in vivo suggests the existence of a feedback regulation mechanism that controls the expression of activity in NK cells.

The functional significance of low NK_a in aplastic patients is uncertain. Long-term monitoring of NK_a in patients undergoing ATG therapy, however, indicates that recovery of NK function correlates well with response to therapy. Patients with aplastic anemia are not abnormally susceptible to viral infections. Although leukemia develops in a small percentage of patients with primary bone marrow failure, the prognosis of this group of patients with severe disease is too poor to allow study of malignant transformation over long periods of time. Nevertheless, the existence of pre-NK cells responsive to lymphokine activation suggests that potentially there exists a reserve of NK cells that might, under appropriate stimulation, confer antiviral or antitumor resistance. Observation of patients who recover hematopoietic function with persistent low NK_a, and of patients with other diseases associated with low NK_a, may permit better definition of a physiologic role in humans for these cells.

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REFERENCES

1. Herberman RB, Ortaldo JR: Natural killer cells: Their role in defense against disease. Science 214:24, 1981

2. Herberman RB (ed): NK Cells and Other Natural Effector Cells. New York, Academic Press, 1982, p 1566

3. Abo, T, Balch CM: A differentiation antigen of human and NK and K cells identified by a monoclonal antibody (HNK-1). J Immunol 127:1024, 1982

4. Timonen T, Ortaldo JR, Herberman RB: Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153:569, 1981

5. Lanier LL, Engelman EG, Gatenby P, Babcock GF, Warner NL, Herzenberg LA: Correlation of functional properties of human lymphoid cell subsets and surface markers phenotypes using multiparameter analysis and flow cytometry. Immunol Rev 74:147, 1983

6. Lanier LL, Phillips JH, Warner NL, Babcock GF: A human natural killer cell associated antigen defined by monoclonal antibody anti-Leu 11a (NKP-15): Functional and two-color flow cytometry analysis. J Leukocyte Biol 35:11, 1984

7. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock G: Subpopulation of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 131:1789, 1983

8. Ortaldo JR, Sharrow SO, Timonen T, Herberman RB: Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. J Immunol 127:2401, 1981

9. Zarling JM, Clouse KA, Biddison WE, Kung PC: Phenotypes of human natural killer cell populations determined with monoclonal antibodies. J Immunol 127:2575, 1981

10. Kasahara T, Djeu J, Dougherty SF, Oppenheim JJ: Capacity of human large granular lymphocytes (LGL) to produce multiple lymphokines: Interleukin 2, interferon and colony stimulating factor. J Immunol 131:2329, 1983

11. Scala G, Allavena P, Djeu J, Kasahara T, Ortaldo JR, Herberman RB, Oppenheim J: Human large granular lymphocytes are potent producers of interleukin-1. Nature 309:56, 1984

12. Herberman RB: Natural cell-mediated immunity against tumors. New York, Academic Press, 1980

13. Hoffman T: Natural killer function in systemic lupus erythematosus. Arthritis Rheum 23:30, 1980

14. Kay NE, Zarling JM: Impaired natural killer activity in patients with chronic lymphocytic leukemia is associated with a deficiency of azurophilic cytoplasmic granules in putative NK cells. Blood 63:305, 1984

15. Dent PB, Fish LA, White JF, Good RA: Chediak-Higashi syndrome. Observation on the nature of the associated malignancy. Lab Inst 15:1634, 1966

16. Sullivan JL, Byron KS, Brewster FF, Purtilo D: Deficient natural killer cell activity in X-linked lymphoproliferative syndrome. Science 210:543, 1980

17. Herberman RB: Natural killer cells and their possible relevance to transplantation biology. Transplantation 34:1, 1982

18. O'Brien TK, Kendra JA, Stephens HAF, Knight RA, Barret AJ: Recognition of marrow elements by natural killer cells: Are NK cells involved in haemopoietic regulation? Br J Haematol 53:161, 1983

19. Holmberg LA, Miller DA, Ault KA: The effect of natural killer cells on the development of syngeneic hematopoietic progenitors. J Immunol 133:2933, 1984

20. Hansson M, Beran M, Anderson B, Kiessling R: Inhibition of in vitro granulopoiesis by autologous and allogeneic human cells. J Immunol 129:126, 1982

21. Mangan KF, Hartnett ME, Matis SA, Winkelstein A, Abo T: Natural killer cells suppress human erythroid stem cell proliferation in vitro. Blood 63:260, 1984

22. Warner JR, Dennert G: Effects of a cloned cell line with NK activity on bone marrow transplants, tumour development and metastasis in vivo. Nature 300:31, 1982

23. Young NS: Aplastic anemia: Research themes, clinical issues. Prog Hematol 12:227, 1981.

24. Gascon P, Zoumbos N, Scala G, Djeu J, Moore J, Young N: Lymphokine abnormalities in aplastic anemia: Implications for the mechanism of action of anti-thymocyte globulins. Blood, 65:407, 1985

25. Zoumbos N, Gascon P, Djeu J, Young N: Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo. Proc Natl Acad Sci USA 82:188, 1985

26. Camitta BM, Thomas ED, Nathan DG, Santos G, Gordon Smith EC, Gale RD, Rappeport JM, Storb R: Severe aplastic anemia: A prospective study of the effect of early marrow transplantation on acute mortality. Blood 48:63, 1976

27. Gascon P, Zoumbos N, Young N: Immunologic abnormalities in patients receiving multiple blood transfusions. Ann Intern Med 100:173, 1984

28. Timonen T, Saksela E: Isolation of human natural killer cells by density gradient centrifugation. J Immunol Methods 36:285, 1980

29. Greco B, Bielory L, Stephany D, Hsu SM, Gascon P,

Nienhuis A, Young N: Antithymocyte globulin reacts with many normal human cell types. Blood 62:1047, 1983

30. Trinchieri G, Santoli D: Anti-viral activity induced by culturing lymphocytes with tumor-derived or virus-transformed cells. Enhancement of human natural killer cell activity by interferon and antagonistic inhibition of susceptibility of target cells to lysis. J Exp Med 147:1314, 1978

31. Kuribayashi K, Gillis S, Kern DE, Henney CS: Murine NK cell cultures: Effects of interleukin-2 and interferon on cell growth and cytotoxic activity. J Immunol 126:2321, 1981

32. Livnat S, Seigneuret M, Storb R, Prentice RL: Analysis of cytotoxic effector cell function in patients with leukemia or aplastic anemia before and after marrow transplantation. J Immunol 124:481, 1980

33. Zoumbos N, Ferris W, Hsu S, Goodman S, Griffith P, Sharrow S, Humphries A, Young N: Analysis of lymphocyte subsets in patients with aplastic anemia. Br J Haematol 58:95, 1984

34. Zoumbos N, Gascon P, Trost S, Djeu J, Young NS: Circulating activated suppressor cells in aplastic anemia. N Engl J Med 312:257, 1985

35. Twomey J, Douglass C, Sharkey O, Jr: The monocytopenia of aplastic anemia. Blood 41:187, 1973

36. Morley A, Holmes K, Forbes I: Depletion of B lymphocytes in chronic hypoplastic marrow failure (Aplastic Anemia). Aust NZ J Med 4:538, 1974

37. Sun D, Lohmann M: Cells with natural killer activity are eliminated by treatment with monoclonal specific anti-macrophage antibody plus complement, in Herberman RB (ed): NK Cells and Other Natural Effector Cells. Academic, New York, 1982, p 243

38. Herberman RB, Holden HT: Natural cell-mediated immunity. Adv Cancer Res 27:305, 1978

39. Minato N, Reid L, Cantor H, Lengyel P, Bloom B: Mode of regulation of natural killer cell activity by interferon. J Exp Med 152:127, 1980

40. Ricardi C, Vose BM, Herberman RB: Regulation by interferon and T cells of IL-2–dependent growth of NK progenitor cells: a limit dilution analysis, in Herberman RB (ed): NK Cells and Other Natural Effector Cells. Academic, New York, 1982, p 909

41. Friedman RM, Vogel SN: Interferons with special emphasis on the immune system. Adv Immunol 34:97, 1983

42. Destefano E, Friedman RM, Friedman-Kien AE, Goedert JJ, Henriksen D, Preble OT, Sonnabend JA, Vilcek J: Acid-labile human leukocyte interferon in homosexual men with Kaposi's sarcoma and lymphadenopathy. J Infect Dis 146:451, 1982

43. Rook H, Masur H, Clifford Lane H, Frederick W, Kasahara T, Macher AM, Djeu Y, Manischewitz F, Jackson L, Fauci AS, Quinnan GV Jr: Interleukin-2 enhances the depressed natural killer and cytomegalovirus-specific cytotoxic activities of lymphocytes from patients with the acquired immune deficiency syndrome. J Clin Invest 72:398, 1983

Mapping of Distinct von Willebrand Factor Domains Interacting With Platelet GPIb and GPIIb/IIIa and With Collagen Using Monoclonal Antibodies

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We have used monoclonal antibodies (M Abs) and proteolytic fragmentation to localize structurally the functional sites of human von Willebrand factor (vWF) responsible for interaction with membrane glycoproteins GPIb, GPIIb/IIIa, and with collagen. SpII (215 kd) and SpIII (320 kd), the *S aureus* V-8 protease homodimeric fragments representing the carboxy-terminal and amino-terminal segments of the vWF subunit, competitively inhibited the binding of multimeric vWF to thrombin-stimulated or ristocetin-stimulated platelets, respectively. Specific saturable binding of each fragment was observed to stimulate platelets appropriately and was inhibited only by selected M Abs that both bound to the specific fragment and inhibited the corresponding function. M Ab 9, which blocks thrombin-induced binding of vWF to platelets, inhibited binding of SpII to platelets and bound to SpII as well as to a dimeric, 86-kd thermolysin fragment composed of 42-kd and 23-kd subunits, each possessing the epitope. Binding of SpII was also inhibited by a M Ab to GPIIb/IIIa. Thus, it appears that a portion of the carboxy-terminal end of vWF contains the ligand site for the GPIIb/IIIa receptor. In contrast, M Ab H9, which blocks ristocetin-induced binding of vWF to platelets, inhibited binding of SpIII to platelets and bound to SpIII as well as to monomeric 33-kd and 28-kd subtilisin fragments. Binding of SpIII to platelets was also inhibited by a M Ab to GPIb. Thus, it appears that a small segment of the amino-terminal part of vWF contains the ligand for the platelet GPIb receptor. The collagen binding site of vWF was localized with M Ab B203, which inhibits vWF interaction with collagen. This M Ab also bound to SpIII as well as to monomeric 26-kd and 23-kd subtilisin fragments. Thus, the third functional site responsible for collagen binding appears to be localized on the amino-terminal portion of vWF, in a linear sequence different from those responsible for interaction with either of the platelet receptors. These assignments of functional sites should facilitate the localization of structural defects of vWF in the various forms of vWD and support the role of vWF as an adhesive protein with multiple interactive sites.

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IN PLASMA, von Willebrand factor (vWF) circulates as a series of ordered multimeric glycoprotein complexes of 1 to ~15 × 10^6 daltons composed of disulfide-linked subunits of ~250 kd.[1-4] vWF mediates platelet adhesion to the subendothelium of the injured vessel wall at high shear rate.[5,6] This physiologic function results from the interaction between specific vWF receptors on platelets[7] and complementary molecular loci on vWF.[8] It has been established that in the presence of ristocetin, platelet membrane glycoprotein Ib (GPIb) is exposed and binds vWF,[7,9-12] whereas membrane glycoprotein complex IIb/IIIa (GPIIb/IIIa) is induced in the presence of more physiologic agonists such as ADP, collagen, and thrombin.[7,13-18] In addition, vWF specifically interacts with collagen in the absence of a known agonist.[19-22]

Proteolytic fragmentation of purified vWF has demonstrated that *S aureus* V-8 protease selectively disrupts the vWF multimers by cleavage of the vWF subunit, resulting in two complementary dimeric fragments.[23,24] One, fragment II, with a mol wt of 215 kd, is composed of two chains with identical mol wts of 110 kd. Both chains contain the carboxy-(C)-terminal end of the vWF subunit.[24,25] The other fragment, referred to as fragment III, appears more heterogeneous, usually migrating as a doublet or a triplet with a major band of mol wt 320 kd unreduced and 170 kd reduced. Both chains of fragment III contain the *N*-terminal portion of the vWF subunit.[24,25] Although the binding domain of vWF to ristocetin-treated, fixed platelets has previously been located on fragment III,[24] the domains binding to thrombin-treated platelets and to collagen have not been yet identified on fragments II or III. Using these purified fragments, here designated SpII and SpIII, we have further localized the functional domains of vWF. For this purpose, 21 previously characterized monoclonal antibodies (M Abs) to vWF were used. Among these, M Ab H9 inhibits ristocetin-induced binding of vWF to platelet GPIb,[26] M Ab 9 blocks thrombin-induced binding of vWF to GPIIb/IIIa,[8] and M Abs B200 through B206 inhibit vWF binding to collagen.[22] The M Ab-defined epitopes have now been identified on the functional vWF domains present in SpII and SpIII fragments. Using M Abs to platelet membrane GPIb and GPIIb/IIIa, we also demonstrated that SpII binds to GPIIb/IIIa induced by thrombin and SpIII to GPIb in the presence of ristocetin. Our results are consistent with the presence of a minimum of three distinct functional domains on vWF, each possessing a ligand structure for a specific receptor on platelets or an acceptor site on collagen. They also permit further development of epitope mapping of vWF combined with assignment of the function to relatively small polypeptide segments of the vWF subunit.

MATERIALS AND METHODS

von Willebrand factor (vWF) and fragmentation. Human vWF was purified from factor VIII concentrates donated by Hyland Therapeutics (Division of Travenol Laboratories Inc, Los Angeles) in the presence of multiple protease inhibitors, including benzamidine, soy bean trypsin inhibitor, diisopropylfluorophosphate (DFP) and *O*-phenanthroline.[3] vWF was isolated by gel filtration on

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Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) as previously described.[3] Contaminants present in the void volume fractions were removed by adsorption on gelatin- and agmatine-Sepharose 4B.[27] Factor VIII was separated from vWF by subsequent agarose gel filtration on Sepharose CL-4B in the presence of 0.5 mol/L of $CaCl_2$. After being desalted on Sephadex G-25, vWF was stored as a 40% saturation ammonium sulfate precipitate[28] at −80 °C. Aliquots were dissolved in 150 mmol/L of NaCl, 25 mmol/L Tris-HCl, pH 7.4 (Tris buffer) and dialyzed against the same buffer. Purified vWF contained 193 vWF:Ag U/mg and 108 vWF R:Co U/mg. It appeared as a typical set of 12 bands by sodium dodecyl sulfate (SDS)-agarose–polyacrylamide gel electrophoresis (PAGE), with mol wts from 1 to 12×10^6 daltons. Contaminating fibrinogen and fibronectin were <0.1%.

Digestion of vWF by *S aureus* V-8 protease (Miles Laboratories Inc, Naperville, Ill) and purification of the fragments were performed as follows: V-8 protease (8 mg) was coupled to Sepharose 2B (10 mL).[29] After equilibration in 50 mmol/L of NaCl, 10 mmol/L of EDTA, and 50 mmol/L of Tris -HCl, pH 7.8, the beads were packed in a column, and purified vWF (24 mg) diluted in the same buffer (15 mL) was digested by recirculating through for 24 hours at 22 °C. After elution, the digest was treated with 1 mmol/L of DFP (final concentration), dialyzed against 50 mmol/L of NaCl, 0.1 mol/L of NaH_2PO_4, pH 5.8, and subjected to ion-exchange chromatography on a 20-mL diethylaminoethanol (DEAE)-Sephacel column equilibrated in the same buffer. The unadsorbed pass-through peak contained a major vWF fragment (SpII). SpII was further purified on a Sephadex G-150 column (1.6 × 90 cm) equilibrated in Tris buffer. The adsorbed protein on DEAE-Sephacel was eluted by a stepwise increase of NaCl concentration from 0.05 to 0.3 mol/L in the starting buffer. This eluted peak contained a major vWF fragment (SpIII) and traces of undigested or partially digested vWF, which were removed by immunoadsorption in Tris buffer onto a selected M Ab to vWF, M Ab 8 (7 mg of IgG) coupled to Sepharose 2B (10 mL). M Ab 8 is specific for an epitope located on both purified and plasmatic vWF and also on SpII but absent from SpIII (see below).

As estimated by SDS-PAGE, purified, unreduced SpII appears as a homogeneous protein with an apparent mol wt of 235 kd by the method of Laemmli[30] or 215 kd by polyacrylamide gradient gel electrophoresis as described by O'Farrel.[31] The same mol wt (110 kd) is observed by both methods following reduction. Purified SpIII appears as a triplet by SDS-agarose-PAGE.[24] A doublet is observed with apparent mol wts of 315 to 270 kd[24] by the Laemmli method and 320 to 280 kd by the O'Farrel method.

As estimated by immunoradiometric assay (IRMA)[32] performed with selected M Abs (IgG) to vWF, purified SpII contained <10 parts of SpIII or vWF per million, and SpIII contained <20 parts of SpII or vWF per million.

Secondary proteolytic fragmentation of SpII and SpIII. Secondary proteolysis of SpII and SpIII was performed at 22 °C in Tris buffer using 400µg of substrate per milliliter and enzyme-substrate ratios from 1:1,000 to 1:25 (wt/wt). SpIII was digested with subtilisin (Boehringer, Mannheim, F.R.G.) for four hours, and SpII was digested with thermolysin (Boehringer) for 24 hours. Proteolysis was terminated with 1 mmol/L of DFP. Thermolysin inhibition was aided by 10 mmol/L of EDTA. Digests were made 1% SDS, heated for 20 minutes at 65 °C in the presence of sucrose (25% wt/vol) and a trace of bromophenol blue and analyzed by SDS-polyacrylamide (3.5% to 16% gradient) slab gel electrophoresis without reduction. Gels were either stained with Coomassie blue or blotted onto nitrocellulose paper (Schleicher and Schüll, Dassel, F.R.G.).[33] After incubation with selected ^{125}I-labeled anti-vWF IgG and extensive washing with 0.15 mol/L of NaCl, the reactive proteins were displayed by autoradiography.

Immunoadsorption with M Abs coupled to Sepharose. Anti-vWF M Ab IgG (1 mg) were coupled to Sepharose 2B (1 mL) as described,[29] and the beads were incubated for two hours with Tris buffer containing 2% bovine serum albumin (BSA, Calbiochem-Behring Corp, La Jolla, Calif) to prevent nonspecific adsorption. Excess of BSA was eliminated by extensive washing with Tris buffer. ^{125}I-SpIII was digested with subtilisin as described above, using an enzyme-substrate ratio of 1:25. A prolonged time (24 hours) of incubation was chosen to eliminate intermediate fragments. Labeled SpII was digested with thermolysin as described. For analysis of binding of reduced-alkylated SpII and SpIII, radioactive digests were reduced with 10 mmol/L of dithiothreitol for one hour at 37 °C and alkylated with 20 mmol/L of iodoacetamide for the same period of time. Untreated or reduced and alkylated digests (~30 µg) were incubated with 0.5 mL of M Ab-Sepharose at 22 °C overnight. The beads were then poured into a column (econo-column, Bio-Rad Laboratories, Richmond, Calif) and washed with Tris buffer containing 2% BSA (~500 mL) until no radioactivity was detected in the eluate. BSA was removed by washing with Tris buffer, and immunoadsorbed ^{125}I-fragments were eluted by suspending the wet cake in 300 µL of 8 mol/L of urea, 1% SDS, 0.125 mol/L of Tris-HCl, pH 6.8, for two hours. After centrifugation for three minutes at 10,000 g (Beckman Microfuge M12, Palo Alto, Calif), the supernatants (100 µL) were analyzed unreduced or reduced (10% β-mercaptoethanol) by SDS-polyacrylamide (gradient) gel or by two-dimensional PAGE.

Characteristics of the M Abs to vWF and to platelet membrane glycoproteins. A panel of 21 M Abs to human vWF was produced, and each was characterized as previously described.[26,34] Typing demonstrated that all were γ 1,K. IgG fractions were purified from ascites fluids as described.[26] In this panel, four types of M Abs were identified: (a) M Ab 9 inhibits the binding of vWF to thrombin-activated platelets[8], (b) M Ab H9 blocks the binding of vWF to ristocetin-treated platelets[26]; (c) M Abs B200, B201, B202, B203, B204, B205, and B206 abolish the interaction of vWF with collagen[22]; and (d) the rest (M Abs 4, 5, 6, 7, 8, 10, 11, B207, B208, 416, 420, and 424) are directed against vWF epitopes that do not demonstrably affect these functional properties. These M Abs were characterized by their reactivity with proteolytic fragments of vWF produced by *S aureus* V-8 protease. Purified vWF and crude V-8 protease digest were analyzed in duplicate by SDS-agarose-PAGE. One gel was stained with Coomassie blue; the other was electroblotted onto nitrocellulose paper and reacted with either ^{125}I-polyclonal (rabbit)[32] or ^{125}I-monoclonal anti-vWF IgG. Results were confirmed by the same electrophoretic/blotting technique using purified SpII and SpIII instead of crude V-8 protease digest and SDS-agarose-polyacrylamide or SDS-polyacrylamide gels. They were in agreement with those obtained by enzyme-linked immunosorbent assay (ELISA)[34] in which purified vWF, SpII, or SpIII were immobilized on polyvinyl 96-well plates and reacted with monoclonal anti-vWF IgG. Among the 21 M Abs tested, eight (including M Ab 9) reacted with SpII, and 13 (including M Abs H9 and B200 through B206) bound to SpIII. None of them reacted with both SpII and SpIII. Results of electroblots of SDS-PAGE showed that the 320-kd and 280-kd bands of SpIII shared at least 13 epitopes, suggesting that the 280-kd fragment derived from the 320-kd species by progressive degradation with V-8 protease.

Two other M Abs (IgG fraction) directed toward platelet membrane glycoproteins were also used in this study. M Ab P2 specific for the associated form of the heterodimeric platelet glycoprotein complex GPIIb/IIIa[35] was kindly donated by Dr J. McGregor, Lyon, France. M Ab AN 51 directed against platelet glycoprotein GPIb[11,36] was a gift from Dr G. Tobelem, Paris. An unrelated M Ab to a mammary tumor glycoprotein (MTGP) was used as control IgG in all experiments.[34]

Radiolabeling. Proteins were labeled with ^{125}I(Amersham International, Amersham, England) using Iodo-Gen (Pierce Chemical Co, Rockford, Ill) as described.[37] Free iodide was removed by gel filtration on a Sephadex G-25 column. Buffer used was either Tris buffer for ^{125}I-IgG or Tyrode's buffer (130 mmol/L of NaCl, 2.6 mmol/L of KCl, 12 mmol/L of $NaHCO_3$, 5.5 mmol/L of glucose, pH 7.2) for ^{125}I-vWF, ^{125}I-SpII, and ^{125}I-SpIII. Labeled preparations of IgG had a specific radioactivity of 8 to 10 mCi/mg. Specific radioactivity varied from 0.2 to 3 mCi/mg for vWF and SpIII and 0.2 to 11 mCi/mg for SpII. The labeled proteins had a comparable behavior in experiments independent of the degree of labeling.

Radioimmunoassays. The presence of fibrinogen and fibronectin in vWF preparations was kindly estimated by radioimmunoassay by Dr G. Marguerie from the same institution.[18] SpII or SpIII purity was estimated by two-site IRMA[32] using pairs of distinct M Abs anti-vWF IgG directed against the same vWF fragment (SpII or SpIII).

Binding to platelets. Platelets were isolated from fresh human blood (60 mL) drawn into citric acid-citrate-dextrose (10 mL) by differential centrifugation and agarose gel filtration on Sepharose CL-2B (60 mL) equilibrated in Tyrode's buffer containing 2% BSA (Tyrode's-BSA buffer), as described.[18,38] ^{125}I-vWF, ^{125}I-SpII, and ^{125}I-SpIII were centrifuged for three minutes at 10,000 g immediately prior to use to analyze binding to platelets, which was performed at 22 °C under nonstirring conditions[18] in 1.5 mL conical polypropylene tubes in a final volume of 0.2 mL of Tyrode's-BSA buffer. Each incubation mixture contained 1 mmol/L of Ca^{2+}, 10^8 platelets per milliliter (20×10^6 cells/0.2 mL), 0.1 U/mL of thrombin (Hoffmann-La Roche, Basel, Switzerland) or 1 mg/mL of ristocetin (Lundbeck Co, Copenhagen) and varying concentrations of ^{125}I-ligand. When thrombin was used as a stimulus, 30 U/mL of hirudin (Diagnostica Stago, Asnières, France) were added two minutes after the thrombin and prior to the labeled protein. Nonspecific binding was performed using identical conditions in the absence of stimulus. In the presence of the stimulus alone but in the absence of platelets, no ^{125}I-ligand was recovered in the pellet.

Binding was quantitated after one hour of reaction. Duplicate aliquots (50 μL) of the platelet suspension were layered onto 300 μL of 25% sucrose in Tyrode-BSA buffer in 400-μL conical polyethylene tubes. After centrifugation for three minutes at 10,000 g, the tips containing the platelets were amputated with a scalpel and associated ^{125}I-ligand was quantitated in a γ counter (1260 multigamma II counter, LKB Instruments SA, Bromma, Sweden). ^{125}I-ligand residual in the supernatant was also determined to control for the total ^{125}I-ligand concentration for each experimental point. Specific binding was calculated by substracting ^{125}I-ligand recovered with nonstimulated platelets from ^{125}I-ligand binding to stimulated platelets. Binding isotherms and double reciprocal plots were generated. For these experiments, the ligand input in each reaction (0.2 mL of ^{125}I-vWF, ^{125}I-SpII, and ^{125}I-SpIII) varied from 2 to 60, 1 to 30, and 0.4 to 60 μg of protein, respectively. Two preparations of ^{125}I-vWF (0.4 and 0.88×10^6 cpm/μg), four preparations of ^{125}I-SpII (0.39, 0.48, 0.59, and 1.2×10^6 cpm/μg), and two preparations of ^{125}I-SpIII (0.36 and 0.49×10^6 cpm/μg) were used in this study.

Platelet bound ^{125} I-ligand was extracted following washing of the pellets with Tyrode's buffer, pH 7.2, and centrifugation (three minutes at 10,000 g) by incubating the platelets in 100 μL of 6 mol/L of urea, 1% SDS, 2 mmol/L of phenylmethane sulfonylfluoride (PMSF), 125 mmol/L of Tris-HCl buffer, pH 6.8 for 30 minutes at 40 °C. Following centrifugation (three minutes at 10,000 g), the supernatant containing >80% of the radioactive material was analyzed by SDS-PAGE (3.5% to 14% gradient) and displayed by autoradiography.

Inhibition of platelet binding of ^{125}I-vWF or ^{125}I-vWF fragments by proteins and M Abs was performed using a modified binding assay. The effect of unlabeled vWF, SpII, or SpIII on ^{125}I-vWF binding was analyzed by incubating stimulated platelets (final concentration 10^8 cells per milliliter) with a constant amount of ^{125} I-vWF premixed with serial dilutions of the unlabeled competitor in Tyrode's-BSA buffer, so that the final concentration of ^{125}I-vWF (5.5×10^6 cpm/μg) was 1 μg/mL (ie, 0.2 μg per incubation mixture). Under these conditions, the total binding of ^{125}I-vWF to thrombin-stimulated platelets performed in the absence of competitor and estimated from 50 μL of incubation mixture was 14.2% of the total available ligand. It was 59.6% when platelet binding was induced with ristocetin. The nonspecific binding in both cases was 0.1%. Essentially it was unmodified by increase in the concentration of SpII or SpIII. This nonspecific binding slightly increased with the concentration of unlabeled vWF and reached 1.9% for 4 mg/mL of vWF.

The effect of M Abs to vWF on ^{125}I-SpII or ^{125}I-SpIII binding was measured by reacting anti-vWF IgG (40 μg/mL) in Tyrode's-BSA buffer for one hour at 22 °C with a constant amount of labeled ligand (2 μg/mL). After centrifugation for three minutes at 10,000 g, the supernatant (100 μL) was incubated with an equal volume of stimulated platelets (20×10^6 cells) (final concentrations: 1 μg/mL ^{125}I-ligand, 10^8 cells per milliliter and 20 μg/mL IgG).

To analyze the effect of M Abs to GPIb or to GPIIb/IIIa, stimulated platelets (20×10^6 cells) were preincubated with IgG (4 μg) in a total volume of 100 μL for 15 minutes at 22 °C prior to addition of 100 μL containing 0.2 μg of labeled ligand (final concentrations: 1 μg/mL ^{125}I-ligand, 10^8 cells per milliliter, and 20 μg/mL IgG). In the absence of competitor, the total binding of ^{125}I-SpII (20×10^6 cpm/μg) to thrombin-stimulated platelets was 0.4% to 0.6% of the total applied and the nonspecific binding was <0.07% (two experiments). The total binding of ^{125}I-SpIII (5.2×10^6 cpm/μg) to ristocetin-treated platelets was 5.4% to 6.1% and the nonspecific binding was <0.15% (two experiments). In all cases, the nonspecific binding remained essentially constant when measured in the presence of competing IgG (20 μg/mL final concentration).

SDS-PAGE. SDS-PAGE was performed in a discontinuous buffer system according to Laemmli[30] or using linear gradients of polyacrylamide as described by O'Farrel.[31] Two-dimensional SDS-PAGE was performed using a Laemmli slab gel (13.5% polyacrylamide)[30] and unreduced samples in the first dimension. After migration, the selected lane was cut, immersed in 25% glycerol, 5% β-mercaptoethanol, 1% SDS, 0.125 mol/L of Tris-HCl buffer, pH 6.8, for 30 minutes at 37 °C and placed at the top of a vertical slab gel prepared for the second dimension. This was composed by a stacking gel (13.5% polyacrylamide) and a gradient (13.5%-20%) polyacrylamide running gel.[31] SDS-agarose-PAGE (0.1% SDS, 6 mol/L of urea, 1.6% acrylamide, 0.75% agarose) using a continuous buffer system (0.1% SDS, 0.1 mol/L of Tris-phosphate buffer, pH 7) was performed as recently described[27] with agarose Sea-Kem HGT(P) (Marine Colloid, Rockland, Me). Mol wt markers included low mol wt standards (Pharmacia), IgG (mol wt 160 kd), fibronectin (mol wt 440 kd) kindly donated by Dr J.M. Freyssinet, Strasbourg, France, and IgM purified from the plasma of a patient with myeloma.[2] Gels were stained with Coomassie blue R, and autoradiography of dried gels was performed at −80 °C, using Kodak-X-Omat S films and Kodak X-Omatic cassettes (Eastman Kodak Co, Rochester, NY).

RESULTS

Effect of purified fragments SpIII and SPII on binding of ^{125}I-vWF to platelets. vWF fragments SpIII and SpII produced by selective cleavage with V-8 protease and purification were analyzed for preservation of the platelet interactive sites by competitive inhibition of the binding of ^{125}I-vWF to ristocetin-treated or thrombin-treated platelets. Inhibition obtained for serial dilutions of each fragment was compared with that produced by unlabeled vWF (Fig 1). Binding of ^{125}I-vWF (1 μg/mL) to fresh ristocetin-treated platelets (10^8 cells per milliliter) was competitively inhibited by SpIII (Fig 1A). Fifty percent of competitive displacement required 280 μg/mL of SpIII as compared with 24 μg/mL of vWF. However, a similar maximum inhibition of more than 90% of the binding of ^{125}I-vWF was observed for both proteins at 2 mg/mL. In contrast, even at 500 μg/mL, SpII was unable to diminish the binding of ^{125}I-vWF to ristocetin-treated platelets. When thrombin-stimulated platelets were analyzed (Fig 1B), complete inhibition of ^{125}I-vWF-binding was observed with SpII or vWF at 1 mg/mL. Half maximum competitive inhibition was produced by SpII at 120 μg/mL or vWF at 20 μg/mL. SpIII (up to 600 μg/mL) had no effect. Thus, each fragment demonstrated a functional capacity to inhibit vWF binding to platelets that was restricted to a single agonist.

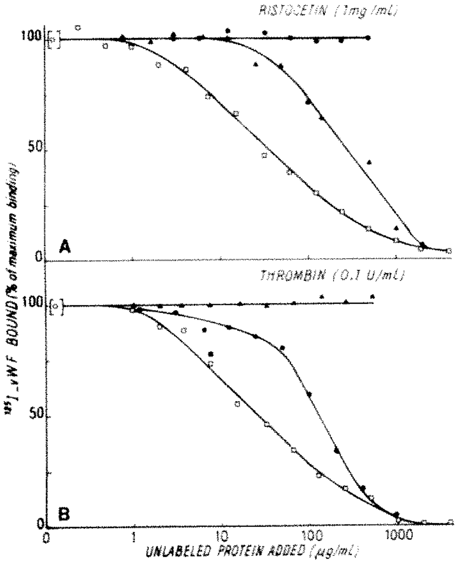

Fig 1. Competitive inhibition analysis of SpII and SpIII. Inhibition of ^{125}I von Willebrand factor (vWF) binding to ristocetin-treated or thrombin treated platelets by unlabeled vWF, SpII, or SpIII was estimated using fresh platelets (10^8/mL) treated with either (A) 1 mg/mL of ristocetin or (B) 0.1 U/mL thrombin and incubated with a mixture of ^{125}I-vWF (1 μg/mL, 5.5 × 10^6 cpm/μg) and serial concentrations of unlabeled protein at 22 °C without stirring. After one hour, 50-μL aliquots (~275,000 cpm) in duplicate were centrifuged through 25% sucrose, and radioactivity in the tip and supernatant of the tubes was counted. In the absence of competitor, total binding was 59.6% of the amount applied in the presence of ristocetin (~164,000 cpm) and 14.2% in the presence of thrombin (~39,000 cpm). Nonspecific binding was 0.1% in both cases and did not vary with the amount of SpII or SpIII added. It increased slightly with vWF concentration. For each point, nonspecific binding was substracted from total binding to obtain the specific binding. Results are expressed as percentages of the specific binding of ^{125}I-vWF in the absence of competitor (○). ^{125}I-vWF was incubated with vWF (□), SpII (●), or SpIII (▲).

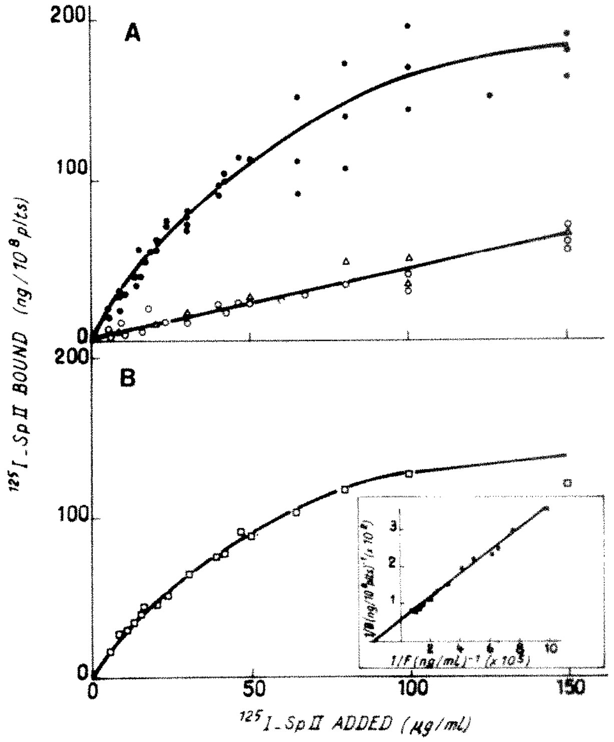

Fig 2. Binding analysis of SpII. The binding of ^{125}I-SpII to thrombin-treated platelets was quantitated using fresh platelets (10^8/mL) stimulated with 0.1 U/mL of thrombin (or 1 mg/mL of ristocetin as control) and incubated with various concentrations of ^{125}I-SpII (5 to 150 μg/mL) at 22 °C without stirring. Nonspecific binding was estimated in the absence of stimulus. After one hour, platelet-associated radioactivity was determined using 50-μL aliquots in duplicate as described in the Materials and Methods section. (A) Results of total and nonspecific binding from four experiments performed with different labeled SpII preparations (0.39, 0.48, 0.59, and 1.2 × 10^6 cpm/μg) and platelets from different donors. The curves represent the least-mean-squares, best-fit lines for all the experimental points. Binding of ^{125}I-SpII was estimated in the presence of thrombin (●), ristocetin (△), or buffer (○). (B) Representative specific binding curve for ^{125}I-SpII in the presence of thrombin (□) obtained by substracting the values of the nonspecific binding curve from those of the total binding curve at the indicated concentrations. Inset contains the double-reciprocal plot derived from the specific binding curve in a range of concentrations of ^{125}I-SpII from 10 to 150 μg/mL.

Binding of SpII to platelets. When thrombin-activated platelets were examined, ^{125}I-SpII was observed to bind in a concentration-dependent and saturable fashion (Fig 2). Total and nonspecific binding curves (Fig 2A) represent the best fit lines for four labeled preparations of SpII. Total binding was low, varying from 20 to 180 ng/10^8 platelets over the range of concentrations used (5 to 150 μg/mL). It was, however, consistently greater than the nonspecific binding, which varied linearly from 4 to 64 ng/10^8 platelets. In the presence of ristocetin, binding of ^{125}I-SpII was indistinguishable from that of the control (Fig 2A). The specific binding of ^{125}I-SpII to thrombin-stimulated platelets is shown in Fig 2B. Values for maximal binding at saturation (n) and apparent dissociation constant (K_d) were further derived from double-reciprocal plots (Fig 2B, inset). The calculated linear regression line has a correlation coefficient r = .99 and extrapolation yields a value of n = 180 ng/10^8 platelets and a K_d of 56 μg/mL. Assuming a mol wt of 215 kd and assuming that only a single ligand site per molecule would participate, 5,100 molecules of SpII bind per platelet with a K_d of 2.6×10^{-7} mol/L. Individual double-reciprocal plots of the four experiments demonstrate a correlation coefficient between 0.96 and 0.99 with a maximal binding of n (±SD) = 5,400 ± 1,500 molecules per platelet and K_d = 3.2 (±1.3) × 10^{-7} mol/L. For comparison, similar analysis performed using two labeled preparations of vWF (final concentrations between 10 and 300 μg/mL) and thrombin-stimulated platelets yielded n = 15,000 to 17,000 molecules per platelet (2.7 to 3.1 μg/10^8 platelets) and an apparent K_d of 2.3 to 2.75 × 10^{-8} mol/L (25.3 to 30.2 μg/mL). For these determinations the mol wt of vWF was considered to be 1.1 × 10^6 (data not shown).

Binding of SpIII to platelets. When ^{125}I-SpIII was used as the ligand, results interpretable as saturable binding to ristocetin-treated platelets were observed (Fig 3) whereas binding to thrombin-stimulated platelets was indistinguishable from the control. Figure 3 shows data for one representative experiment performed with ^{125}I-SpII (0.36 × 10^6 cpm/μg). Total binding in the presence of ristocetin increased from 0.12 to 2.4 μg/10^8 platelets as SpIII concentration increased from 2 to 300 μg/mL. Nonspecific binding linearly increased to 0.5 μg/10^8 platelets at the highest concentration of SpIII. A double-reciprocal plot (Fig 3, inset) was derived from the specific binding curve for concentrations ranging from 10 to 300 μg/mL. The linear regression line had a correlation coefficient of r = .99, and extrapolation yielded a maximal binding at saturation of 2.5 μg/10^8 platelets, with an apparent K_d of 69.8 μg/mL. Assuming a mol wt of 320 kd and a single interactive site per molecule, ~47,000 molecules of SpIII bind per platelet, with a K_d of 2.2×10^{-7} mol/L. Similar values (n = 54,000 molecules per platelet with K_d = 3.6 × 10^{-7} mol/L) were obtained in experiments using a different ^{125}I-SpIII preparation (0.49 × 10^6 cpm/μg).

Under similar conditions, vWF (final concentrations ranging from 10 to 300 μg/mL) was found to bind specifically to ristocetin-treated platelets (two experiments) with a maximum of 44,000 to 45,800 molecules per platelet (8 to 8.4 μg/10^8 platelets) and an apparent K_d of 1.7 to 2.3 × 10^{-8} mol/L (19.4 to 25.4 μg/mL) assuming a mol wt of 1.1 × 10^6 for vWF (data not shown).

Characterization of platelet-bound ^{125}I-SpIII and ^{125}I-SpII. Platelet-bound SpIII and SpII used as the radiolabeled ligands were analyzed by SDS-PAGE and autoradiographic display following extraction from the platelet pellet. Figure 4 compares their electrophoretic mobility to that of purified labeled fragments. When unreduced, purified SpIII

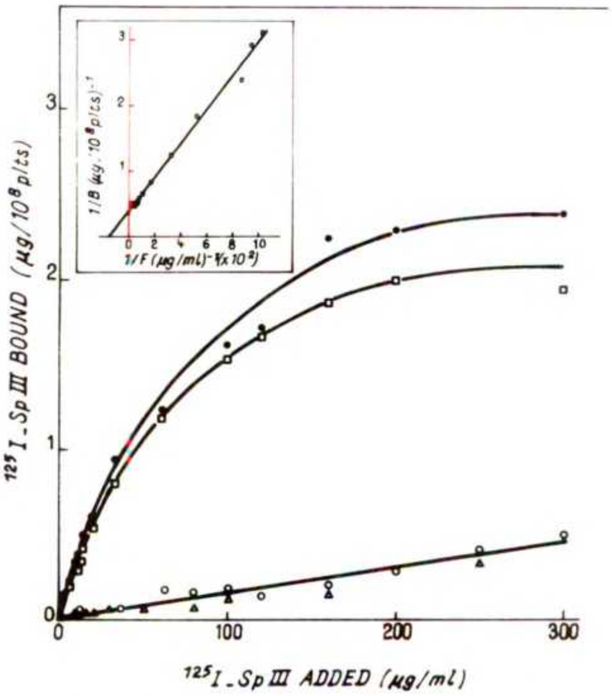

Fig 3. Binding analysis of SpIII. The binding of ^{125}I-SpIII (2 to 300 μg/mL to platelets (10^8/mL) treated with 1 μg/mL of ristocetin (or 0.1 U/mL of thrombin as control) was quantitated using a labeled preparation of SpIII with a specific activity of 0.36 × 10^6 cpm/μg. Nonspecific binding estimated in the absence of stimulus (○) was substracted from total observed binding in the presence of ristocetin (●) to derive specific binding (□). Total binding in the presence of thrombin (0.1 U/mL) is also indicated (△). Inset is a double-reciprocal plot derived from the specific binding values in the presence of ristocetin over a range of concentrations of ^{125}I-SpIII from 10 to 300 μg/mL.

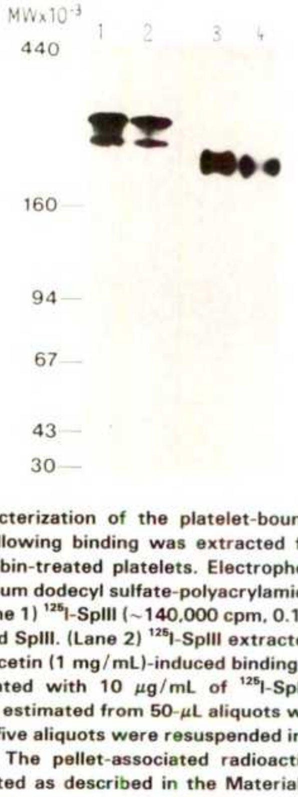

Fig 4. Characterization of the platelet-bound protein. The labeled ligand following binding was extracted from ristocetin-treated or thrombin-treated platelets. Electrophoresis was performed using sodium dodecyl sulfate-polyacrylamide (3.5% to 14% gradient) gel. (Lane 1) ^{125}I-SpIII (~140,000 cpm, 0.1 μg) mixed with 10 μg of unlabeled SpIII. (Lane 2) ^{125}I-SpIII extracted from platelet pellets after ristocetin (1 mg/mL)-induced binding. Platelets (10^8/mL) were incubated with 10 μg/mL of ^{125}I-SpIII (1.38 × 10^6 cpm/μg). Binding estimated from 50-μL aliquots was 33,500 cpm. The pellets from five aliquots were resuspended in buffer, pooled, and centrifuged. The pellet-associated radioactivity (~150,000 cpm) was extracted as described in the Materials and Methods section. Recovered radioactivity (~120,000 cpm) was loaded on the gel. (Lane 3) ^{125}I-SpII (~120,000 cpm, 0.02 μg) mixed with 10 μg of unlabeled SpII. (Lane 4) ^{125}I-SpII extracted from platelet pellets after thrombin (0.1 U/mL) -induced binding. Platelets (10^8/mL) were incubated with 20 μg/mL of ^{125}I-SpII (~6 × 10^6 cpm/μg). Binding estimated from 50-μL aliquots was 15,000 cpm. The pellets from five aliquots were washed, and associated radioactivity (~67,000 cpm) was extracted as described above; recovered radioactivity (~55,000 cpm) was loaded on the gel. Position of the mol wt markers is indicated at left (fibronectin, mol wt 440 kd; IgG, mol wt 160 kd).

migrated as a doublet (mol wt 320 to 280 kd) with a major band at 320 kd (lane 1), and SpII appeared as a single band with a mol wt of 215 kd (lane 3). Analysis of the material bound to ristocetin-treated platelets showed that both bands comprising SpIII reacted with platelets and retained the same mobility after extraction (lane 2). Similarly, the material bound to thrombin-stimulated platelets had the mobility of purified SpII (lane 4). In neither analysis were other bands detected.

Inhibition of binding of SpII and SpIII to platelets by specific M Abs. Inhibition of specific binding of labeled SpII or SpIII to platelets by selected M Abs specific for different platelet surface receptors were used as a means to confirm the specificity of binding. M Abs specific for either GPIb or GPIIb/IIIa were used at 125 nmol/L (20 μg/mL). As shown in Table 1, when ristocetin-treated platelets were preincubated with IgG AN 51 specific for GPIb, binding of ^{125}I-SpIII was reduced to <3% of the control values obtained with buffer or control IgG at 125 nmol/L. Incubation of platelets with IgG P2 specific for GPIIb/IIIa did not modify ^{125}I-SpIII binding, which remained at 98% of the control values. Conversely, using the same final concentration of M Ab IgG, maximum binding of ^{125}I-SpII to thrombin-stimulated platelets was reduced to 19% when IgG P2 was used, whereas it was unmodified in the presence of AN 51 (Table 1).

Inhibition of labeled SpIII or SpII binding by anti-vWF M Abs H9 and 9 IgG was analyzed using the same final concentration of 20 μg/mL (Table 1). Interaction of SpIII with platelets in the presence of ristocetin was completely abolished by M Ab H9. This binding was not attenuated by M Ab 9 (99% of the control values). With thrombin-treated platelets, ^{125}I-SpII binding was not inhibited by M Ab H9 but was virtually abolished by M Ab 9 (residual binding 2%).

Table 1. Inhibition of ^{125}I-SpIII or SpII Binding to Stimulated Platelets by M Abs (IgG) to GPIb, GPIIb/IIIa, and vWF

Competing Antibody (125 nmol/L)	Residual Binding (%)	
	^{125}I-SpIII	^{125}I-SpII
Buffer	100	100
AN 51	2.5	90
P2	98	19
H9	0	104
9	99	2
Anti-MTGP	95	98

M Abs, monoclonal antibodies; vWF, von Willebrand factor; MTGP, mammary tumor glycoprotein.

Preincubation of platelets or ^{125}I-ligand with buffer or 125 nmol/L (20 μg/mL final concentration) of IgG was performed as described in the Materials and Methods section. Incubations were performed using a final concentration of 1 μg/mL of labeled ligand and 10^8 stimulated platelets per milliliter. Stimuli were either ristocetin (1 mg/mL) for SpIII or thrombin (0.1 U/mL) for SpII. Results are the mean of two determinations in triplicate. In two experiments using ^{125}I-SpIII, total count was ~260,000 cpm/50-μL aliquots, and average total binding (buffer) was 14,000 to 15,800 cpm. The nonspecific binding (~400 cpm) was subtracted to estimate residual binding. In two experiments using ^{125}I-SpII, total counts were ~1 × 10^6 cpm/50-μL aliquots, and average total binding (buffer) was 4,000 to 6,000 cpm. The nonspecific binding was ~700 cpm. M Abs AN 51 and P2 are specific for GPIb and GPIIb/IIIa, respectively. M Abs H9 and 9 block binding of vWF to platelets in the presence of ristocetin and thrombin, respectively. Anti-MTGP is used as control M Ab IgG.

Localization of GPIb- and collagen-binding domains of vWF on SpIII. Secondary fragments of purified SpIII obtained by limited proteolysis (four hours) with subtilisin were characterized by SDS-PAGE. SpIII migrated as a doublet of mol wt 320 to 280 kd (Fig 5A). At low concentrations of subtilisin, it was fragmented into four primary intermediates of 215, 170, 135, and 94 kd. Increase of enzyme concentration led to final products of 135-, 90-, 45-, 40-, 35-, and 30-kd fragments as well as minor amounts of 78-, 65-, and 50-kd fragments.

Reaction of the separated and blotted subtilisin digest of SpIII with labeled M Ab H9 (Fig 5B) demonstrated that the primary 215-kd and 170-kd fragments expressed the vWF epitope H9 related to binding to GPIb. This epitope was also detected on the 135-kd and 90-kd fragments, which were present in trace amounts at the early stage and increased during the course of digestion with subtilisin. Finally, the H9 epitope appeared to be present, although poorly reactive with M Ab H9, on a 30-kd terminal digestion fragment.

Reaction of the same subtilisin digest of SpIII with labeled M Ab B203 produced an entirely different pattern (Fig 5C). The vWF epitope B203 related to interaction with collagen was strongly expressed on a 94-kd fragment. This fragment was degraded by further proteolysis, leading to intermediates of 78, 60, and 50 kd. Finally, a small fragment with a mol wt of 45 kd containing the B203 epitope was produced in the terminal digest (Fig 5C).

In other experiments using ^{125}I-SpIII and prolonged (24-hour) digestion by subtilisin, species of mol wt >67 kd were

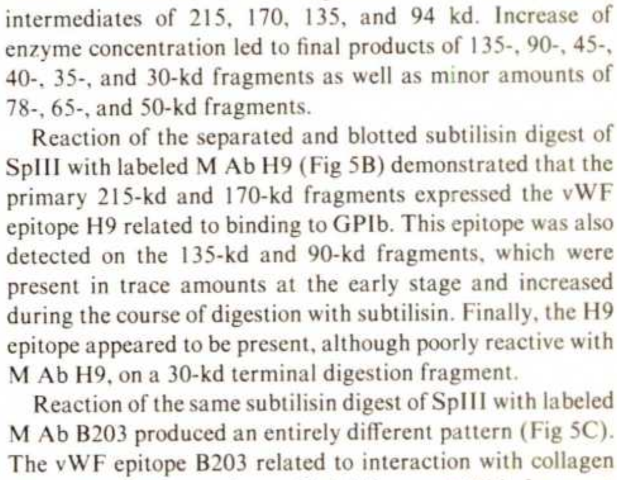

Fig 5. Polyacrylamide gel electrophoretic analysis of the products of digestion of SpIII by subtilisin. SpIII (400 μg/mL) was treated for four hours at 22 °C, pH 7.4, with the enzyme at different enzyme-substrate ratios (from left to right: 0, 1:1,000, 1:500, 1:100, 1:50, 1:25). After electrophoresis in sodium dodecyl sulfate-polyacrylamide (3.5% to 16% gradient) gel, proteins were stained with Coomassie blue (A) or transferred onto nitrocellulose paper and revealed with ^{125}I-IgG H9 (B) or ^{125} I-IgG B203 (C). Position of the mol wt markers is indicated at left.

no longer detectable. The terminal digest (Fig 6A) appeared as a series of bands from 20 to 33 kd (unreduced). When subjected to immunoadsorption on M Ab H9 and eluted, two bands of 33 and 28 kd were recovered. Similarly, two bands of 26 and 33 kd different from those isolated by M Ab H9 were isolated by immunoadsorption on M Ab B203. These species were analyzed for their subunit composition by two-dimensional PAGE (Fig 7). The two species isolated on M Ab H9 (Fig 7A) had an identical mol wt when analyzed unreduced (first dimension) or after reduction (second dimension). Reduction of the two fragments isolated on M Ab B203 (Fig 7B) resulted in a slight shift of mol wt from 26 and 23 kd to 24 and 28 kd, respectively. In neither case could any other spot be detected by autoradiography.

To determine the role of the quaternary structure in the function of epitopes H9 and B203, we performed immunoadsorption after reduction and alkylation of the digest (Fig 6B). Reduced digest showed a series of bands in the same range of mol wt as that observed before reduction (Fig 6A), suggesting that most of the fragments were monomeric. The reduced and alkylated polypeptides immunoadsorbed by M Ab H9 were identical to those observed using unreduced fragments with two main bands of 33 and 28 kd. Similarly, the two species immunoadsorbed by M Ab B203 (Fig 6A) still retained their reactivity toward the M Ab B203 after reduction and alkylation (Fig 6B).

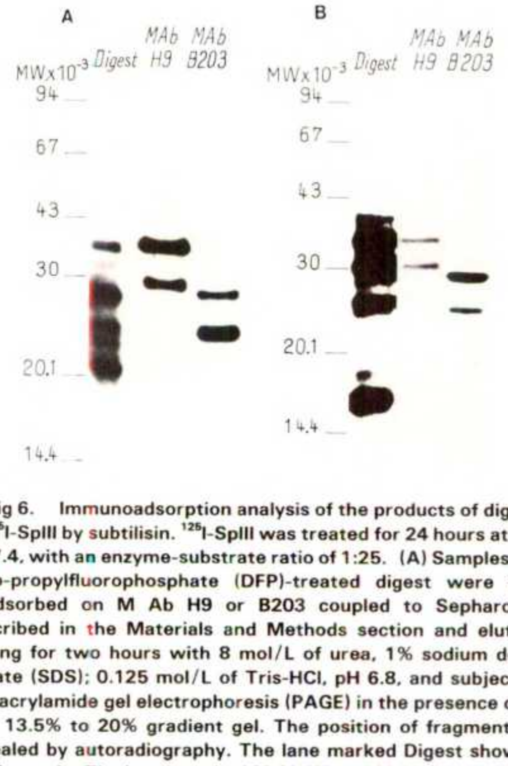

Fig 6. Immunoadsorption analysis of the products of digestion of ^{125}I-SpIII by subtilisin. ^{125}I-SpIII was treated for 24 hours at 22 °C, pH 7.4, with an enzyme-substrate ratio of 1:25. (A) Samples of the diiso-propylfluorophosphate (DFP)-treated digest were immunoadsorbed on M Ab H9 or B203 coupled to Sepharose as described in the Materials and Methods section and eluted by mixing for two hours with 8 mol/L of urea, 1% sodium dodecyl sulfate (SDS); 0.125 mol/L of Tris-HCl, pH 6.8, and subjected to polyacrylamide gel electrophoresis (PAGE) in the presence of SDS in a 13.5% to 20% gradient gel. The position of fragments was revealed by autoradiography. The lane marked Digest shows the total sample. The lanes marked M Ab H9 and M Ab B203 show the materials immunoadsorbed on the respective M Abs. (B) Samples of the DFP-treated digest were reduced with 10 mmol/L of dithiothreitol for one hour at 37 °C and alkylated with 20 mmol/L of iodoacetamide before immunoadsorption on M Ab H9 and B203. The lane marked Digest shows the total sample after reduction and alkylation. The lanes marked M Ab H9 and M Ab B203 show the materials reduced and alkylated and then immunoadsorbed on the respective M Abs. Position of markers is indicated at left in A and B.

Localization of GPIIb/IIIa-binding domain of vWF on SpII. Proteolytic fragmentation of purified SpII using thermolysin was characterized by SDS-PAGE. In addition to SpII of mol wt 215 kd, three bands were produced by proteolysis when analyzed by protein staining (Fig 8A). By means of increasing concentrations of thermolysin, a 200-kd intermediate was detected. A 125-kd fragment was produced, followed by 120-kd and 98-kd intermediates. At the latter stage of proteolysis, only fragments with mol wts of 86, 64, and 48 kd remained.

By immunoblotting with M Ab 9, epitope 9 related to binding of vWF to GPIIb/IIIa was present on SpII as well as on the 200-kd species (Fig 8B). It was also demonstrated on the 125-kd and 120-kd intermediates and at the end of the digestion on a 86-kd fragment. Epitope 9 was no longer demonstrable on the 64-kd and 48-kd terminal fragments.

When ^{125}I-SpII was proteolytically cleaved to smaller polypeptides by thermolysin at 1:25 (enzyme-SpII), the pattern of fragments was identical to that observed for cleavage of unlabeled SpII. Three major bands of 86, 65, and 48 kd resulted. Immunoadsorption on M Ab 9 confirmed that only the 86-kd fragment was bound (data not shown). Analysis of the digest after reduction and alkylation showed a series of bands from 42 to 14 kd (Fig 9). The 86-kd species immunoadsorbed to M Ab 9 and then eluted and reduced appeared as two bands of 42 and 23 kd (Fig 9, central lane). The same bands were isolated from the digest when immunoadsorption on M Ab 9 was performed after reduction and alkylation (Fig 9, right lane), confirming the localization of epitope 9.

DISCUSSION

von Willebrand Factor, an adhesive protein important in the mediation of physiologic hemostasis, appears to interact with the platelet as well as with the subendothelium through three independent defined binding sites. Two of the vWF binding structures or receptors are major glycoproteins on the platelet surface membrane.[7-18] Binding of vWF to GPIb is induced by the agonist ristocetin, whereas the second binding structure, which is mediated by the heterodimeric complex GPIIb/IIIa, can be functionally induced by a variety of agonists, including ADP and thrombin. The third binding reaction, as yet structurally undefined, involves binding of vWF to collagen of the subendothelium.[19-22] Several lines of evidence suggest that distinct domains of the vWF molecule may be involved in the two independent platelet interactions and in the binding to collagen. By enzymatic degradation of vWF, one group[39] demonstrated that intact subunits were not required for vWF-mediated aggregation of ristocetin-stimulated platelets and that the domain corresponding to that activity, which has been related to the binding of vWF to GPIb, was located on a 116-kd fragment. More recently, using M Abs to unrelated epitopes of vWF and toward GPIb or GPIIb/IIIa, Nokes et al[8] reported that thrombin-induced binding of vWF to GPIIb/IIIa corresponded to a different domain of the molecule than did that involved in ristocetin-induced binding to

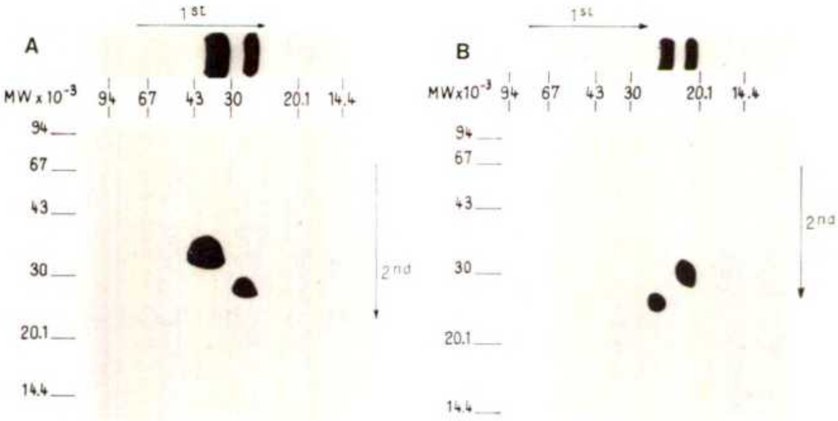

Fig 7. Two-dimensional polyacrylamide gel electrophoretic (PAGE) analysis of ^{125}I-SpIII digested by subtilisin for 24 hours and immunoadsorbed with (A) M Ab H9 or (B) B203 followed by autoradiographic display. Digestion and immunoadsorption were performed as described in the Materials and Methods section. The immunoadsorbed fragments were electrophoresed in duplicate on a 13.5% polyacrylamide slab gel. One lane was cut and reduced in 25% glycerol, 5% β-mercaptoethanol, 1% sodium dodecyl sulfate, 0.125 mol/L of Tris-HCl buffer, pH 6.8 for 30 minutes at 37 °C. The gel was then placed on top of a 13.5% to 20% polyacrylamide gradient slab gel and electrophoresed. The second lane was displayed by autoradiography and used as marker of the first dimension. The arrows indicate the direction of the migration. Position of the markers run in parallel in the first and second dimension are indicated.

GPIb. Similarly, Sixma et al[28] have used an independent series of M Abs specific for vWF accompanied by tryptic proteolysis of the protein. They confirmed the presence of the binding domain of vWF for ristocetin-stimulated platelets on a 116-kd fragment and localized the collagen interacting domain to an apparently unrelated 48-kd species.

In the present study, we used purified vWF fragments to identify the binding domain of the protein to the platelet surface GPIIb/IIIa receptor and to compare its location with that of the other two interacting regions, ie, the GPIb interactive site and the collagen binding site. Limited hydrolysis of vWF by *S aureus* V-8 protease leads to the formation of two major fragments, SpII and SpIII, which we have characterized and purified.[24] SpII possesses the C-terminal portion of the vWF subunit and is a 215-kd homodimer composed of two polypeptide chains of 110 kd. The second fragment, SpIII, contains the N-terminal portion of the subunit. It is heterogeneous by SDS-PAGE in that it migrates as a doublet containing a predominant 320-kd homodimer composed of two chains of 170 kd and a second

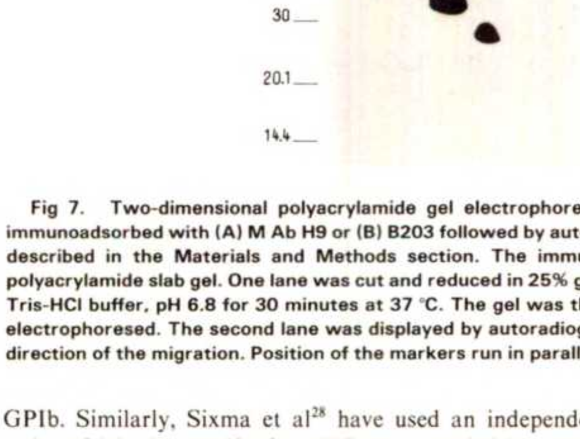

Fig 8. Polyacrylamide gel electrophoretic analysis of the products of digestion of SpII by thermolysin. SpII (400 μg/mL) was treated for 24 hours at 22 °C, pH 7.4, with different enzyme-substrate ratios (from left to right: 0, 1:1000, 1:500, 1:100, 1:50, 1:25). Electrophoresis was performed on sodium dodecyl sulfate-polyacrylamide (3.5% to 16% gradient) gel. (A) Coomassie blue staining. (B) electroblotting and reaction with ^{125}I-IgG M Ab 9. Position of markers is indicated at left.

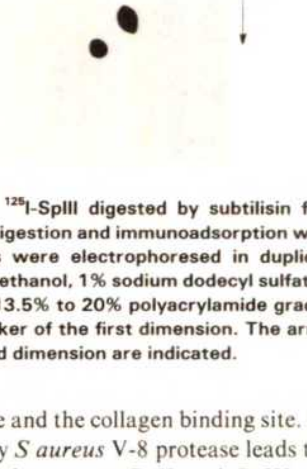

Fig 9. Immunoadsorption analysis of the proteolytic fragments of SpII. ^{125}I-SpII was hydrolyzed with thermolysin for 24 hours at 22 °C, pH 7.4 with an enzyme-substrate ratio of 1:25. The lane marked Digest shows the total sample after reduction with 10% β-mercaptoethanol and analysis on a 3.5% to 16% polyacrylamiae gradient gel in sodium dodecyl sulfate. The central lane shows the material immunoadsorbed to immobilized M Ab 9, eluted as described in Fig 6, reduced by 10 mmol/L of dithiothreitol, and alkylated. The right lane shows the material reduced and alkylated prior to immunoadsorption on M Ab 9. Position of markers is indicated at left.

less abundant species of 280 kd composed of the 170-kd chain associated to a 104-kd polypeptide.[24] Our results using M Abs to a series of distinct epitopes of vWF indicate that the two bands comprising SpIII share at least 13 different epitopes. In addition, these bands demonstrate a similar subunit composition (170 kd), identical behavior by ion-exchange chromatography,[24] and similar binding properties to platelets. Thus, we assume that the 280-kd band is derived from the major 320-kd species by a secondary proteolytic digestion of a less susceptible bond in one of the 170-kd subunits.

The homodimeric structure of the SpII and SpIII fragments and the mol wt of the reduced forms which total that of the subunit within the accuracy of the method suggest that they are complementary and encompass virtually the entire length of the vWF subunit. This was further illustrated by kinetic studies, demonstrating the coordinant appearance of SpII and SpIII in concert with the disappearance of the intact vWF subunit.[24] Our present results with M Abs to vWF confirm the complementary, nonoverlapping structure of the two primary fragments. All of our M Abs to vWF recognized either SpII (eight antibodies) or SpIII (13 antibodies); none recognized both fragments. In addition, we observed that M Abs B200 through B206 and H9 (which inhibit interaction of vWF with collagen[22] and with GPIb,[26] respectively) bind to epitopes localized on SpIII, whereas M Ab 9 (which abolishes binding of vWF to platelet surface GPIIb/IIIa[8]) recognizes SpII. Thus, the three biologically active domains of vWF appear to be present after digestion by V-8 protease and are structurally independent of one another.

The functional activity of the SpIII and SpII fragments was clearly observed when they were examined for specific saturable binding to platelets in the presence of either ristocetin or thrombin; this was confirmed by independent competitive inhibition of the binding of intact vWF by the purified fragments. Using metabolically active platelets, we confirmed previous observations obtained with formalin-fixed platelets[24] showing that the interaction of vWF with platelets in the presence of ristocetin is fully abolished by SpIII whereas SpII has no effect. In addition, we observed that binding of vWF to thrombin-stimulated platelets was completely inhibited by SpII but not by SpIII. Results of direct binding assay of ^{125}I-labeled fragments to platelets indicated that they bound only to the appropriately treated platelets, and binding appeared saturable. This is consistent with the interpretation that the inhibition of vWF binding resulted from a specific interaction of SpII or SpIII with the platelets rather than with vWF only or nonspecifically with the platelet. Thus, thrombin induced a specific site for SpII on the platelet membrane, and ristocetin induced a specific site for SpIII. The two binding sites were saturable, and temporal dependence was observed, reaching a maximum at 60 minutes (data not shown). Specificity was illustrated by the failure of the complementary vWF fragment to compete with each other. Characterization of the platelet-bound material by gel electrophoresis confirmed that SpII and SpIII bound specifically and were not degraded on the platelet surface.

Identification of the receptors for SpIII and SpII on the platelet membrane was analyzed using M Abs specific for GPIb and GPIIb/IIIa. When platelets were exposed to an excess of M Ab AN 51 specific for GPIb, complete inhibition of SpIII binding to ristocetin-treated platelets was observed. In contrast, no noticeable effect of M Ab AN 51 on SpII or vWF interaction with thrombin-treated platelets was detected. Thus, in the presence of ristocetin, the platelet site for SpIII appears to be dependent on GPIb, and the competitive inhibition of ^{125}I-vWF binding by SpIII indicates that the sites for both ligands are identical. Additional evidence was obtained with use of anti-vWF M Ab H9, as our results demonstrated that binding to the corresponding epitope on SpIII or vWF produced the same effect, ie, a total inhibition of binding to GPIb. We conclude that SpIII contains the same binding domain as the multimeric vWF protein for the same receptor, namely GPIb. However, comparison of the steric hindrance induced by M Ab AN 51 on vWF binding (50% inhibition)[11] and on SpIII binding (100% inhibition) suggested a strikingly different accessibility for both ligands to the receptor.

Immunochemical methods were also used to establish that the receptor for SpII is the GPIIb/IIIa heterodimeric complex on thrombin-stimulated platelets. M Ab P2 specific for the associated functional GPIIb/IIIa complex[35] on platelets inhibited binding of labeled SpII. In contrast, this M Ab had no effect on binding of SpIII or vWF to ristocetin-treated platelets. We found that the interaction of either vWF or SpII with GPIIb/IIIa was inhibited to the same extent (80%) by P2 (not shown), suggesting that this M Ab may act by steric hindrance rather than by direct occupancy of the common binding site for both ligands on GpIIb/IIIa. In addition, the complete inhibition of both SpII and vWF by saturation of epitope 9 with M Ab 9 demonstrates the identity of the platelet GPIIb/IIIa-related domain on SpII and vWF.

Comparison of inhibition of ^{125}I-vWF binding by vWF, SpIII, or SpII gives clear evidence that at low concentrations the fragments possess a diminished capacity to bind to their receptor as compared with vWF. As estimated at 50% displacement, vWF appears ten- and 40-fold more effective than SpIII on a weight or a molar basis, respectively. vWF appears 6 and 30 times more effective than SpII in the same types of analysis. In addition, steeper slopes were observed when fragments were tested. This is consistent with the hypothesis that a homogeneous binding site is present on the fragments, contrasting with the presence of more heterogeneous sites of both high and low affinity on vWF. Comparative analysis of the binding to platelets was attempted, assuming a single binding site per molecule of vWF (mol wt 1.1×10^6), SpII (215 kd) and SpIII (320 kd). When labeled SpII or SpIII was used, the specific binding was low as compared with the total amount of ^{125}I-ligand applied, and binding analysis required high ligand concentration. The apparent affinity of the fragments was about tenfold less than that for vWF using the same appropriate platelet agonist.

The importance of the size of the multimeric vWF for binding to platelets has been established by other investigators.[40] It was also recently suggested that proteolytic cleavage of vWF may induce major changes in vWF structure.[41] Thus, our results may be interpreted as a consequence of the reduced size and/or the modified conformation of the fragments as compared with that of the vWF multimers. The low relative percentage of binding of SpII and SpIII may also result in significant errors as reflected by the disparity in estimation of K_d from one experiment to the other. This has also required use of ligands of relatively high specific radioactivity, which may have influenced their apparent affinity for platelets as compared with the native protein. Finally, to address the possible role of differences in the multimeric vWF structure, the same preparation was used as a source of SpII and SpIII for all the binding experiments. It possessed both high-affinity and low-affinity binding sites. Of the 45,000 binding sites found in the presence of ristocetin, 5,900 were of high affinity, with K_d of 0.51×10^{-9} mol/L; in the presence of thrombin, 8,500 of the 17,000 sites had a K_d of 11.4×10^{-9} mol/L (data not shown). These values and the characteristics of the low-affinity sites are consistent with those previously established by others using the same assumptions.[9,15,40,42-44] In addition, we demonstrated that the pattern of digestion of purified vWF by V-8 protease was independent of the starting material used to purify vWF (fresh plasma, intermediate purity concentrates from Kabi or CRTS, Lille, or the concentrate used in this study). We cannot totally exclude, however, that the relative decrease of the proportion of very high mol wt multimers in our starting material may have had some influence on the results. Thus, although double-reciprocal plots derived from binding curves of fragments resulted in significant linear correlations, the K_d and the estimated number of binding sites should be considered as estimates, as already emphasized for vWF by other authors.[43,44]

The apparent close spatial relationship between epitopes H9, 9, and B203 and sites of vWF involved in its interactions with GPIb, GPIIb/IIIa, and collagen, respectively, led us to use the three M Abs for identification of the functional domains on fragments obtained from SpIII or SpII by secondary digestion. Proteolysis of SpIII with subtilisin led to an early segregation of H9 and B203 epitopes at low enzyme concentration, indicating their residence on different parts of SpIII and thus their independence.

Our experiments of immunoadsorption on M Abs H9 and B203 performed using native or reduced fragments from prolonged digestion of SpIII with subtilisin confirmed the previous observation from Sixma et al[28] that a native quarternary structure is not required to detect epitopes related to GPIb and collagen binding domains. A terminal monomeric fragment of 28 kd possessing the H9 epitope was intact and functional after complete reduction. Similarly, epitope B203 present on a terminal monomeric fragment was detected before (23 kd) and after reduction (28 kd). The shift in apparent mol wt after reduction is assumed to reflect a significant conformational effect of intrachain disulfide bonds in this fragment.

Extensive proteolytic degradation of SpII using thermolysin led to the formation of three fragments (mol wts 86, 65, and 48 kd). Only the 86-kd species contained epitope 9 related to vWF binding to GPIIb/IIIa. This fragment was composed of 42-kd and 23-kd chains, both possessing the epitope for M Ab 9 after reduction. Because the presence of repetitive epitopes 9 along the 110-kd subunit of SpII is unlikely, we assume that the 86-kd fragment may appear as a dimer (2 × 42 kd), and that the 23-kd subunit derives from a 42-kd species by an additional proteolytic cleavage between two interchain disulfide bridges.

The present study demonstrates that the three functional sites of vWF involved in binding to GPIb, GPIIb/IIIa, and collagen, respectively, are functionally and structurally distinct. They are located on relatively small fragments that are separable following proteolytic digestion of the homodimeric primary fragments of vWF, SpII, and SpIII. The GPIb ligand site and the collagen binding domain reside on different segments of the SpIII fragment, the N-terminal part of the vWF subunit chain. In contrast, GPIIb/IIIa ligand domain is on the SpII fragment, the C-terminal portion of the vWF subunit. We do not yet know the relative positions of the respective domains and their smallest structural units within SpII or SpIII. Such information should follow from more precise mapping of the epitopes on SpII and SpIII within the context of the primary structure.

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REFERENCES

1. Counts RB, Paskell SL, Elgee SK: Disulfide bonds and quaternary structure of factor VIII/von Willebrand factor. J Clin Invest 62:702, 1978

2. Fass DN, Knutson GJ, Bowie EJW: Porcine Willebrand factor: A population of multimers. J Lab Clin Med 91:307, 1978

3. Meyer D, Obert B, Pietu G, Lavergne JM, Zimmerman TS: Multimeric structure of factor VIII/von Willebrand factor in von Willebrand's disease. J Lab Clin Med 95:590, 1980

4. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of factor VIII/von Willebrand factor. Blood 57:1140, 1981

5. Weiss HJ, Turritto VT, Baumgartner HR: Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. J Lab Clin Med 92:750, 1978

6. Baumgartner HR, Tschopp TB, Meyer D: Shear rate dependent inhibition of platelet adhesion and aggregation on collagenous surfaces by antibodies to human factor VIII/von Willebrand factor. Br J Haematol 44:127, 1980

7. Ruggeri ZM, De Marco L, Gatti L, Bader R, Montgomery RR: Platelets have more than one binding site for von Willebrand factor. J Clin Invest 72:1, 1983

8. Nokes TJC, Mahmoud NA, Savidge GF, Goodall AH, Meyer D, Edgington TS, Hardisty RM: von Willebrand factor has more than one binding site for platelets. Thromb Res 34:361, 1984

9. Kao KJ, Pizzo SV, McKee PA: Demonstration and character-

ization of specific binding sites for factor VIII/von Willebrand factor on human platelets. J Clin Invest 63:656, 1979

10. Moake JL, Olson JD, Troll JH, Tang SS, Funicella T, Peterson DM: Binding of radioiodinated human von Willebrand factor to Bernard-Soulier, thrombasthenic and von Willebrand's disease platelets. Thromb Res 19:21, 1980

11. Ruan C, Tobelem G, McMichael AJ, Drouet L, Legrand Y, Degos L, Kieffer N, Lee H, Caen JP: Monoclonal antibody to human platelet glycoprotein I. II. Effects on human platelet function. Br J Haematol 49:511, 1981

12. Coller BS, Peerschke EI, Scudder LE, Sullivan CA: Studies with murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: Additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 61:99, 1983

13. Fujimoto T, Ohara S, Hawiger J: Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor VIII/von Willebrand factor on human platelets. J Clin Invest 69:1212, 1982

14. Fujimoto T, Hawiger J: Adenosine diphosphate induces binding of von Willebrand factor to human platelets. Nature 297:154, 1982

15. Ruggeri ZM, Bader R, De Marco L: Glanzmann thrombasthenia: Deficient binding of von Willebrand factor to thrombin-stimulated platelets. Proc Natl Acad Sci USA 79:6038, 1982

16. Di Minno G, Shapiro S, Catalano PM, De Marco L, Murphy S: The role of ADP secretion and thromboxane synthesis in factor VIII binding to platelets. Blood 62:186, 1983

17. Gralnick HR, Williams SB, Coller BS: Fibrinogen competes with von Willebrand factor for binding to the glycoprotein IIb/IIIa complex when platelets are stimulated with thrombin. Blood 64:797, 1984

18. Pietu G, Cherel G, Marguerie G, Meyer D: Inhibition of von Willebrand factor–platelet interaction by fibrinogen. Nature 308:648, 1984

19. Nyman D: Interaction of collagen with the factor VIII antigen-activity von Willebrand factor complex. Thromb Res 11:433, 1977

20. Legrand YJ, Rodriguez-Zeballos A, Kartalis G, Fauvel F, Caen JP: Adsorption of factor VIII antigen-activity complex by collagen. Thromb Res 13:909, 1978

21. Santoro SA: Adsorption of von Willebrand factor/factor VIII by the genetically distinct interstitial collagens. Thromb Res 21:689, 1981

22. Girma JP, Kalafatis M, Pietu G, Lavergne JM, Edgington TS, Meyer D: Mapping of functional domains of human von Willebrand factor using monoclonal antibodies. Thromb Haemost 54:59, 1985 (abstr)

23. Girma JP, Pietu G, Chopek MW, Edgington TS, Meyer D: Localization of distinct Willebrand factor domains interacting with ristocetin or thrombin stimulated platelets using proteolysis and monoclonal antibodies. Circulation 70:836A, 1984 (abstr)

24. Girma JP, Chopek MW, Titani K, Davie EW: Limited proteolysis of human von Willebrand factor by *S aureus* V-8 protease: Isolation and partial characterization of a platelet-binding domain. Biochemistry (in press)

25. Titani K, Ericsson LH, Kumar S, Dorsam H, Chopek MW, Fujikawa K: Chemical characterization of human von Willebrand factor. Circulation 70:837A, 1984(abstr)

26. Meyer D, Baumgartner HR, Edgington TS: Hybridoma antibodies to human von Willebrand factor. II. Relative role of intramolecular loci in mediation of platelet adhesion to the subendothelium. Br J Haematol 57:609, 1984

27. Chopek MW, Girma JP, Fujikawa K, Davie EW, Titani K: Human von Willebrand factor: A multivalent protein composed of identical subunits. Biochemistry (in press)

28. Sixma JJ, Sakariassen KS, Stel HV, Houdijk WPM, In der Maur DW, Hamer RJ, de Groot PG, van Mourik JA: Functional domains on von Willebrand factor. Recognition of discrete trypic fragments by monoclonal antibodies that inhibit interaction of von Willebrand factor with platelets and with collagen. J Clin Invest 74:736, 1984

29. March SC, Parikh I, Cuatrecasas P: A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal Biochem 60:149, 1974

30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680, 1970

31. O'Farrel PH: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007, 1975

32. Ardaillou N, Girma JP, Meyer D, Lavergne JM, Shoa'i I, Larrieu MJ: "Variants" of von Willebrand's disease. Demonstration of a decreased antigenic reactivity by immunoradiometric assay. Thromb Res 12:817, 1978

33. Towbin H, Stachelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979

34. Meyer D, Zimmerman TS, Obert B, Edgington TS: Hybridoma antibodies to human von Willebrand factor. I. Characterization of seven clones. Br J Haematol 57:597, 1984

35. McGregor JL, Brochier J, Wild F, Follea G, Trzeciak MC, James E, Dechavanne M, McGregor L, Clemetson KJ: Monoclonal antibodies against platelet membrane glycoproteins. Characterization and effect on platelet function. Eur J Biochem 131:427, 1983

36. McMichael AJ, Rust NA, Pilch JR, Solchynsky R, Morton J, Mason DY, Ruan C, Tobelem G, Caen JP: Monoclonal antibody to human platelet glycoprotein I. I. Immunological studies. Br J Haematol 49:501, 1981

37. Fraker PJ, Speck JC: Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1, 3, 4, 6-tetrachlor-3α, 6α-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978

38. Marguerie GA, Plow EF, Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254:5357, 1979

39. Martin SE, Marder VJ, Francis CW, Loftus LS, Barlow EH: Enzymatic degradation of the factor VIII–von Willebrand protein: A unique tryptic fragment with ristocetin cofactor activity. Blood 55:848, 1980

40. Gralnick HR, Williams SB, Morisato DK: Effect of the multimeric structure of the factor VIII/von Willebrand factor protein on binding to platelets. Blood 58:387, 1981

41. Hamilton KK, Fretto LJ, Grierson DS, McKee PA: Effects of plasmin on von Willebrand factor multimers. Degradation in vitro and stimulation of release in vivo. J Clin Invest 76:261, 1985

42. Morisato DK, Gralnick HR: Selective binding of factor VIII/von Willebrand factor protein to human platelets. Blood 55:9, 1980

43. Miller JL, Kupinski JM, Castella A, Ruggeri ZM: von Willebrand factor binds to platelets and induces aggregation in platelet-type but not Type IIB von Willebrand disease. J Clin Invest 72:1532, 1983

44. Harrison RL, McKee PA: Comparison of thrombin and ristocetin in the interaction between von Willebrand factor and platelets. Blood 62:346, 1983

Autologous Bone Marrow Transplantation Using Marrow Incubated With Asta Z 7557 in Adult Acute Leukemia

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The sensitivity of human myeloblastic leukemic (CFU-L) and normal hemopoietic stem cells (CFU-GM and BFU-e) to Asta Z 7557 (INN Mafosfamide) was studied with regard to autologous bone marrow transplantation (ABMT) with cleansed marrow for consolidation therapy in adult patients with acute leukemia (AL) in remission. Establishment of the dose-response curves for CFU-GM (n = 37), BFUe (n = 11), and myeloblastic CFU-L (n = 9) demonstrated a wide range of sensitivity from patient to patient for all three progenitors. Whereas CFU-L, CFU-GM, and BFU-e grown in semisolid cultures disclosed similar sensitivities to Asta Z 7557, long-term culture (LTC) studies (n = 41) indicated a higher resistance of early progenitors. In an effort to achieve a maximum tumor cell kill and yet spare a sufficient amount of normal stem cells to ensure consistent engraftment, we defined the optimal dose for marrow cleansing as the dose sparing 5% CFU-GM (LD_{95}). This dose was established from a preincubation test (PIT) realized on a 10-mL marrow aspirate taken 15 days before marrow collection in each individual patient. Twenty-four adult patients while in remission of AL (20 in complete remission, four in partial remission) were consolidated by cyclophosphamide 60 mg/kg × 2 and total body irradiation at 10 Gy followed by ABMT with marrow cleansed by Asta Z 7557 according to the specification described above. Patients were divided in two groups: group 1, unfavorable prognosis (11 patients); group 2, standard prognosis [13 patients in first complete remission (CR)]. All patients engrafted on leukocytes (median day for recovery to 10^9/L: day 30), patients with ALL recovered faster than patients with ANL (median day 19 *v* 34). Similarly, recovery of platelets to 50.10^9/L occurred sooner in patients with ALL (median day 67, range day 23 through 90) whereas three patients with acute nonlymphoblastic leukemia (ANLL) in group 2 had to be supported with platelet transfusions for more than one year. In group 1, six patients had recurrent tumor within six months; three patients died from toxicity with no evidence of tumor. Two patients are still disease-free with a short follow-up (nine and ten months). In group 2, two patients died from toxicity with no evidence of leukemia three and 16 months post-ABMT. One patient with a M5 ANLL and one patient with ALL relapsed at six and 15 months, respectively. Nine patients have remained in CR or are disease-free with a median follow-up of 22 months. Eight of these patients have been in CR for more than one year (13+, 14+, 17+, 22+, 26+, 26+, 26+, and 27+ months). We conclude that (a) Asta Z can be used in vitro to cleanse the marrow of patients with leukemia following an individual adjustment of the dose, which might enhance benefit-risk ratio; and (b) ABMT with marrow cleansed by Asta Z 7557 is of little benefit to patients with poor-risk leukemias. On the other hand, patients with standard risk factors may derive some improvement in terms of unmaintained disease-free survival.

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ALLOGENEIC BONE marrow transplantation has recently improved the prognosis of acute leukemias[1-3] in patients with Hl-A identical siblings.

Autologous bone marrow transplantation (ABMT) may offer a similar chance to most patients with no available donor. However, one of the major impediments to effective ABMT may be the persistence of leukemic cells in the marrow, even though it is collected when the patient is in complete remission (CR). Therefore, it is generally assumed although not yet proven[4] that the collected marrow should be cleansed before reinfusion. Following the initial report on the efficacy of cyclophosphamide (CY) derivatives to eliminate residual marrow leukemic cells in the Brown Norway myelocytic leukemia (BNML) rat leukemia model,[5] we studied the in vitro effect of ASTA Z 7557 (INN Mafosfamide) (Professor N. Brock, Asta Werke, Bielefeld F.R.G.) on human leukemic and normal hematopoietic stem cells[6] and initiated a therapeutic trial using ABMT with marrow incubated with this drug as a consolidation therapy in adult patients with acute leukemia in remission. There were two goals: (a) to treat the marrow of each individual patient with the highest possible dose of Asta Z 7557 for maximum antileukemic activity, which would also spare enough normal stem cells for successful engraftment; and (b) to assess the potential benefit of high-dose consolidation followed by ABMT with marrow cleansed as described above, in terms of duration of remission and disease-free survival.

MATERIAL AND METHODS

In Vitro Studies

Progenitor cell culture in semisolid media. Blast cells were taken from the marrow of nine patients with acute nonlymphoblastic leukemia (ANLL) at initial diagnosis. More than 70% were peroxidase-positive blasts. "Normal" marrow hematopoietic progenitors were derived from 37 patients [14 acute lymphoblastic leukemia (ALL), 23 ANLL] with acute leukemia in complete remission).

Leukemic cell progenitors from myeloblastic leukemia (CFU-L) were cultured in methylcellulose according to the technique of

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Chang et al[7] and were scored on day 7. Cytological examination of removed colonies confirmed that they consisted of pure blast cells.

Granulocyte-macrophage progenitors (CFU-GM) were assayed in semisolid agar by a modification of the technique of Pike and Robinson[8] and scored after incubation for ten days at 37 °C in a humidified 5% CO_2 atmosphere.

Erythroid progenitors (BFU-e) were cultured in methylcellulose by a modification of the technique of Iscove and Sieber[9] and scored after incubation for 14 days at 37 °C in a humidified 5% CO_2 atmosphere. The erythroid nature of the cells in colonies was confirmed by examination of picked colonies stained with May-Grunwald-Giemsa.

For each assay, 2.10^5 mononuclear cells were cultured in triplicate. Results were expressed in terms of the number of colonies per milliliter of marrow.

Continuous liquid cultures. Long-term liquid cultures were established using a modification of the technique of Dexter et al.[10] Bone marrow buffy coat cells (10^6) from normal donors were suspended in 1 mL of α medium (GIBCO Biocult) supplemented with 20% fetal calf serum (FCS); 5 mL of the suspension was put into Falcon 25-cm^2 tissue culture flasks and incubated at 37 °C in a 5% CO_2 atmosphere. Cultures were maintained by weekly demi-depopulation with addition of fresh medium. Under those conditions, a uniform adherent layer spread within three to four weeks. This primary culture was then depopulated of all suspension cells and irradiated at 30 Gy. A new allogenic inoculum of bone marrow cells was added to the adherent layer. Cultures were maintained by replacing all of the medium each week, without demi-depopulation. At the same time, suspension cells were assayed for CFU-GM as described above.

Cell treatment. Bone marrow cells were incubated with increasing doses of Asta Z 7557 for 30 minutes at 37 °C in a water bath with frequent agitation. Then, they were washed once in Hanks' balanced salt solution (HBSS) and cultured. Buffy coat cells were obtained from bone marrow samples spun at 2,500 rpm for ten minutes adjusted to 2.10^7 cells per milliliter in autologous plasma and TC 199 solution, with a final hematocrit of 5%. The sensitivity of leukemic and normal progenitor cells to Asta Z 7557 was evaluated by the diminution of their number.

For the preincubation test (PIT), a 10-mL marrow aspirate was taken 15 days before bone marrow collection. The sensitivity of remission CFU-GM and BFU-e to increasing doses of Asta Z 7557 was studied as described above, and a dose-response curve was established for each patient. The optimal dose for incubation of the marrow was defined as the dose sparing 5% CFU-GM on the PIT.

Clinical Trial

ABMT was done in the consolidation mode as soon as possible in patients still in remission, after analysis of results of in vitro studies. The protocol was approved by the Saint-Antoine Human Subject Review Committee.

Patients. From January 1983 to January 1985, 24 adult patients entered the study. Oral consent was obtained from all. Patients were divided into two groups.

Group 1 consisted of 11 patients with advanced disease and/or poor prognosis criteria (Table 1, patient numbers 1 to 11). Four patients were in partial remission (PR) following multiple unsuccessful attempts to achieve CR. Two of these patients had additional poor-risk features (presence of the Philadelphia chromosome in one, second relapse of acute megakaryoblastic leukemia in the other). Five patients were in second CR. Two patients were in first CR; one had an undifferentiated acute leukemia (AL) secondary to breast cancer and one, although in CR at the time of autograft, had previously developed a leukemic meningitis. Before ABMT, all of these patients had been heavily pretreated with multiple combinations of drugs.

Group 2 consisted of 13 patients in first CR with standard-risk prognosis factors (Table 2, patient numbers 21 through 33). For these patients, the median duration between CR and ABMT was five months (range 2 to 23 months). In four patients, the decision to autograft was made after a long interval (more than six months) after diagnosis. One patient (UPN 21) had failed induction therapy and gone into CR under a different maintenance regimen. One patient had chronic hepatitis for ten months (UPN 29). Two patients (UPNs 28 and 32) had an M5 acute megakaryoblastic leukemia (AML) for which results of current conventional chemotherapy protocols in our institution were poor (0% survival at five years). Furthermore, in two of these patients (UPNs 28 and 29), marrow collection was delayed because of the detection of a minor contaminating blast cell population by routine marrow surveillance on several occasions. Prior to consolidation with ABMT, patients with ALL were treated according to the French national protocol (arm B) delivered as follows: one induction course combining vincristine (VCR) 1.2 mg/m^2 on days 1 and 5, rubidazone (RBZ) 450 mg/m^2 on day 1, prednisone 80 mg/m^2/day and cytosine-arabinoside (ARA-C) 100 mg/m^2/day by continuous infusion for five consecutive days (days 1 through 5). This induction course was then followed by two to three courses of "3 A," combining Adriamycin (ADR) 40 mg/m^2 on day 1; ARA-C, 60 mg/m^2/day subcutaneously (SC) from day 3 through day 7; and L-Asparaginase (L-ASP) : 1,000 U/kg from day 8 through day 12.

Patients with ANLL were put into CR by one or two courses of ARA-C (100 mg/m^2 q 12 hours on days 1 through 9) + daunorubicin (DNR) (45 mg/m^2/day on days 1 through 3) or the same as above + 6 thioguanine (6 TG) (100 mg/m^2 q 12 hours on days 1 through 9) and VCR 1.4 mg/m^2 on day 1. Two to three courses of ARA-C + 6 TG for four days were then delivered on a monthly schedule. Patient 28, who was grafted late in the course of the disease, received multiple additional courses of chemotherapy on a rotating schedule (ARA-C, DNR, VM 26, CCNU, 6 TG, Methyl GAG).

Finally, all patients (ALL and ANLL) prior to ABMT, received central nervous system (CNS) prophylaxis with six weekly intrathecal administrations of methotrexate (15 mg total dose) + methylprednisolone (20 mg) and cranial irradiation at 15 Gy.

Collection of marrow, incubation with Asta Z 7557, and cryopreservation. A total volume of 600 to 1,300 mL of bone marrow (BM) was collected from the posterior iliac crests, in each patient, under general anesthesia. A volume corresponding to 0.5 10^8 nucleated BM cells per kilogram was saved and directly cryopreserved to serve as a back-up marrow. The remaining volume was further processed for incubation with Asta Z 7557. The buffy coat was collected on a hemonetics H30 cell separator and adjusted to a final cell concentration of 2.10^7 cells per milliliter with TC 199 medium with a final hematocrit of 5%. The suspension was finally incubated with Asta Z 7557 (at the concentration previously established from the PIT as sparing 5% CFU-GM) for 30 minutes in a water bath at 37 °C, with gentle shaking. Following incubation, the BM suspension was immediately cooled and centrifuged at a temperature of 4 °C to block the action of the drug abruptly. After two washes, the BM cells were then resuspended in irradiated autologous plasma (40 Gy) and TC 199 medium and finally frozen with 10% dimethylsulfoxide (DMSO) in two or three Teflon-capton DF 1000 Gambro bags (Gambro Dialysatoren, GMBH, F.R.G.) using a Nicool 316 programmed biological freezer (CFPO, Sassenage, France), designed to ensure a constant cooling rate of −1 °C/min and to compensate for the heat of fusion without inducing a

Table 1. ABMT With Marrow Cleansed by ASTA Z 7557 in Poor-Risk AL

Patient No.	Diagnosis: FAB Classification, Sex/Age	Status at Time of ABMT (Poor-Risk Factors)	Dose of ASTA Z/2.10^7 Cells (μg)	Surviving Fraction (%)		Dose of Marrow Infused* (CFU-c/kg)	Time to Recovery to (days)		Posttransplant Outcome and Follow-up
				CFU-c	BFU-e		WBC > (1,000/μL)	PLTS > (50,000/μL)	
1	ANLL4 F/42	PR, first (Ph^1 chromosome)	80	21	34	1,840	21	—	Persisting leukemia; death on day 90
2	Undifferentiated F/45	CR, first (Second leukemia on breast cancer)	60	47	100	2,256	18	—	Relapse of leukemia and breast cancer on day 80; death on day 100
3	Megakaryoblastic leukemia F/50	PR, third	130	0	0	0	34	—	CR at time of death from pulmonary aspergillosis and CMV on day 100
4	ANLL1 F/17	CR, second	85	29	—	1,770	45	—	Relapse on day 135; CMV infection; death on day 150
5	ANLL2 M/26	CR, second	50	2	2	674	30	—	Relapse on day 60
6	ANLL2 F/13	CR, second	50	28	26	13,700	34	55	Relapse on day 135
7	T ALL M/27	PR, first	120	10	—	2,266	18	—	Toxic death on day 60 from acute VOD, DIC, and renal failure; no evidence of tumor (WBCs 6,500/μL)
8	T ALL F/15	CR, second	105	0.8	—	74	30	50	Relapse on day 120
9	ANLL2 F/36	CR, first (CNS involvement)	60	0.3	—	65	32	—	CR at time of death from severe GI bleeding on day 330
10	ALL1 M/27	PR, first	110	0.2	22	70	19	27	CR 10+ mo
11	ANLL3 F/41	CR, second	105	0	0	0	19	90	CR 9+ mo

ABMT, autologous bone marrow transplantation; AL, acute leukemia; FAB, French-American-British; ANLL, acute nonlymphoblastic leukemia; ALL, acute lymphoblastic leukemia; PR, partial remission; CR, complete remission; CNS, central nervous system.

*Evaluated after incubation with ASTA Z and before cryopreservation.

Table 2. ABMT With Marrow Purged by ASTA Z 7557 in AL in First Remission (Standard Risk)

Patient No.	Diagnosis: FAB Classification, Sex/Age	Dose of ASTA Z/2.10^7 Cells (μg)	Surviving Fraction (5%)		Dose of Marrow Infused* (CFU-c/kg)	Time to Recovery to (days)		Duration of Remission Pretransplant (mo)	Outcome and Follow-up Posttransplant (mo)	Total Survival (mo)
			CFU-c	BFU-e		WBC > 1,000/μL	PLTS > 50,000/μL			
21	ALL1 M/27	50	8	52	600	22	32	11	CR 26+	38+
22	ALL2 M/25	80	4	2	968	19	23	4	CR 15 systemic relapse	‡28
23	ALL2 M/38	140	0.4	1	245	17	67	5	CR 22+	28+
24	ALL2 F/25	55	3	2	1033	11	90	5	CR 17+	23+
25	ALL1 M/55	90	0	—	0	28	90	4	CR 14+	23+
26	ANLL2 F/39	15†	5	4	521	32	485	3	CR 27+	35+
27	ANLL1 M/42	70	6	48	580	34	457	5	CR 26+	32+
28	ANLL5 F/34	50	6	7	863	33	63	23	CR 26+	56+
29	ANLL4 M/40	50	2	62	106	40	—	11	CR 16	‡29 death from viral infection
30	ANLL2 M/30	90	0	—	0	36	180	2	CR 13+	22+
31	ANLL4 M/31	90	0.5	—	39	26	—	6	CR 3	‡12 death from liver VOD
32	ANLL5 F/44	95	0.6	8	256	47	150	8	CR 6 hepatic relapse	‡16 death from viral infection with lung fibrosis
33	ANLL1 M/43	90	1	3	284	29	156	3	CR 8+	11+

ABMT, autologous bone marrow transplantation; AL, acute leukemia; FAB, French-American-British; ALL, acute lymphoblastic leukemia; ANLL, acute nonlymphoblastic leukemia; CY, cyclophosphamide; TBI, total body irradiation; CR, complete remission.

All patients received CY 60 mg/kg/day × 2 and TBI (10 Gy).

*Evaluated after incubation with ASTA Z and before cryopreservation.

†Ficoll gradient separation of mononuclear cells.

‡Deceased.

subsequent detrimental increase in the third slope of the cooling curve.[11] The cleansed marrow was then stored in the gas phase of liquid nitrogen at a temperature constantly below −190 °C. Cell counts, CFU-GM and BFU-e evaluations were done at all steps of the procedure. The numbers of residual progenitor cells following incubation with Asta Z were known for each individual patient prior to ABMT. In three instances (patients 21, 26, and 27), because recoveries in CFU-GM following the cleansing procedure did not match the prediction from the PIT, a second BM collection was done and incubation was repeated to achieve our target of 5% residual CFU-GM. ABMT could therefore be done in most cases with the best available cleansed marrow, in keeping with the design of the study.

High-dose consolidation, ABMT. The basic heavy consolidation treatment consisted of CY 60 mg/kg × 2 (along with MESNA: 60% of the dose of CY), followed after a 2-day rest period by total body irradiation (TBI) at 10 Gy with a dose rate at the source of <6 cGy/min, and lung shielding at 8 Gy. This basic regimen was given to all patients except patient 2 who received only 3 Gy on the left hemithorax because of previous irradiation at 45 Gy for breast cancer. Patients 3 and 7 (group 1) received a higher intensification with additional ARA-C 3 g/m^2 for four and three infusions. Patient 31 (group 2) received additional VP 16: 150 mg/m^2 × 3. The bags of frozen cleansed marrow were thawed rapidly in a water bath at 37 °C and infused immediately, within 36 hours after TBI. Day 0 was defined as the day of marrow infusion.

Analysis of data and statistical methods. Sensitivity of leukemic and normal hematopoietic stem cells to Asta Z 7557 was studied by measuring (in triplicate, each dose of Asta Z 7557 from 20 to 160 μg/mL by increments of 10 μg) the proportion of remaining stem cells as compared with the untreated marrow. For each patient, because of a sigmoid evolution of this proportion in relation to the dose of Asta Z 7557, a probit transformation was used to linearize the relationship, taking into account the number of replicates used for each dose.[12] For each curve, the linear slope and the deviation from linearity were tested by classical F test.[12] Each curve was characterized by its linear slope and the doses necessary to achieve a 50% (LD_{50}) and a 95% (LD_{95}) reduction of the initial stem cell pool.

For these three parameters of each individual marrow, the mean and SD were determined. We tested the heterogeneity of the data by using components of variance analysis.[13] For this purpose, the variability of each parameter among patients was compared with the common estimate of this variability within each patient by classical F test.[12] Comparison between sensitivities of various stem cell categories was performed with the nonparametric Wilcoxon or paired Wilcoxon test,[14] to avoid disturbance due to the nonnormality of the parameters. For continuous liquid culture data, correlation between the maximum CFU-GM regenerations and the percentage of residual CFU-GM at the time of initiation of culture was studied by the Spearman nonparametric test.[14]

The kinetics of recovery of hemopoiesis were studied in particular to evaluate the adverse effects of Asta Z 7557 treatment of the marrow on the normal stem cell pool; the day of engraftment was defined as the first day with a WBC count $>1.10^9/L$. The probability to reach this point by a given posttransplant day was examined by the Kaplan-Meier method.[15] Platelet recovery was considered adequate when the platelet count reached $50.10^9/L$ with no further need for support.

Following ABMT, patients with no evidence of tumor by usual criteria were classified as being in CR. The disease-free survival posttransplant in the two groups of patients was evaluated by the Kaplan-Meier method, and comparison between the two curves was done using the Log-rank test.

RESULTS

In Vitro Studies

Sensitivity of CFU-L, CFU-GM, and BFU-e to in vitro treatment with Asta Z 7557. The slope of the linear decrease was greater for CFU-L when compared with both CFU-GM ($P < .05$) and BFU-e ($P < .001$) (Table 3). No difference was observed for the LD_{50}. When the LD_{95} was studied, BFU-e appeared less sensitive than CFU-GM ($P < .01$) or CFU-L ($P < .05$).

A major observation was the existence of wide variations in sensitivity from patient to patient. The variability among patients appeared to be greater than the variability of results within individuals, by study both of the LD_{50} and the LD_{95} variances ($P < .01$ for CFU-GM with 36 and 270 df; $P < .05$ for BFU-e with 10 and 94 df). In our view, this observation supported an individual adjustment of the dose of Asta Z 7557.

Determination of the optimal dose of Asta Z 7557. Because of the inability at least with routine laboratory technique to evaluate the risk of injuring pluripotential stem cells when destroying 100% CFU-GM, we defined the optimal dose of Asta Z for the in vitro treatment of the whole BM as the dose sparing 5% residual CFU-GM. As shown in Fig 1, the DL_{95} definition zone for most patients (75%) spreads over 14 to 30 μg/2.10^7 buffy coat cells. In our experience, this allows an accurate and reliable determination of the appropriate dose on a PIT: in a series of 32 marrow-cleansing procedures (24 patients of the clinical trial and 8 additional cases), residual CFU-GM were evaluated following incubation of the whole BM marrow with the predetermined dose, and the results were compared with those given by the PIT.

In 25 cases, in which all the technical parameters were adequate, the actual CFU-GM recovery measured after treatment of collected marrow was correlated with the expected recovery predicted by the PIT curve ($r = .473$, $P < .03$) and accuracy of the recovery was ± 4%. In the other seven cases, discrepancies were observed that could be retrospectively attributed to an excessive hematocrit at time of bone marrow incubation (4 cases), an inappropriately low temperature of the incubator (2 cases), and presence of cell aggregates (1 case).

Figure 1 shows that if we decided to incubate all marrows

Table 3. Sensitivity of CFU-L, CFU-GM, and BFU-e to ASTA Z 7557

	CFU-L ($n = 9$) Median (Range)	CFU-GM ($n = 37$) Median (Range)	BFU-e ($n = 11$) Median (Range)
S*	0.073	0.046	0.024
	(0.033-0.149)	(0.023-0.119)	(0.019-0.035)
LD_{50}†	35.1	39.1	44.4
	(18.8-51.5)	(17.9-84.6)	(11.8-92.4)
LD_{95}†	62.6	75.3	122.1
	(47.4-93.7)	(37.7-132.9)	(66.2-179.0)

*Slope (percentage of destruction per microgram of Asta Z 7557) characterizing the linear decrease in residual stem cells in relation to increase of Asta Z 7557.

†Doses (microgram of ASTA Z 7557 per 2.10^7 cells) resulting in a 50% (LD_{50}) and a 95% (LD_{95}) destruction of initial stem cells.

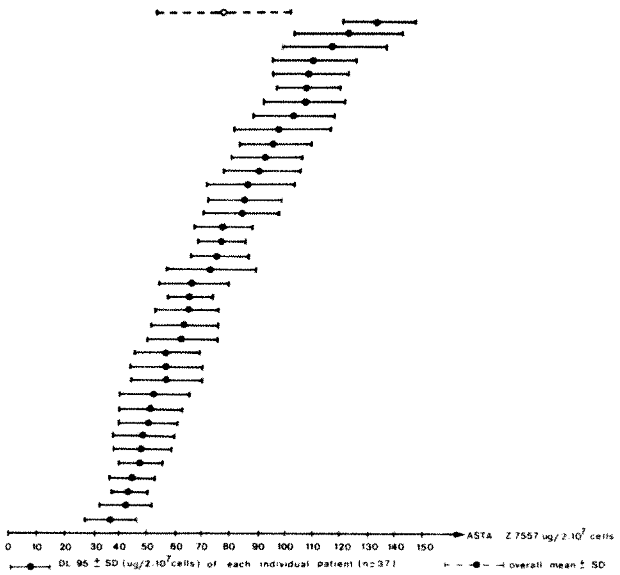

Fig 1. Individual susceptibility of granulocyte-macrophage colony-forming units (CFU-GM) to Asta Z 7557.

with a standard dose of Asta Z, rather than to adapt the dose to each patient, the best choice of dose would be 70 $\mu g/2.10^7$ cells, which would fit our objective to leave 5% residual CFU-GM in 35% of the patients. With this standard dose, 38% of the patients would have their marrow treated less intensively than possible and 27% would be overtreated, with possible damage to the normal pluripotent stem cell pool.

Continuous liquid culture. Twenty-eight BMs from patients with AL in CR were treated with various doses of Asta Z 7557 (ranging from 25 to 140 $\mu g/2.10^7$ buffy coat cells). Three series were defined: series 1 (n = 7) included marrows treated with moderation still producing a median CFU-GM recovery of 39% (22% to 58%); series 2 (n = 13) included marrows treated according to our protocol with a median CFU-GM recovery of 3% (1.5% to 9%); series 3 (n = 8) included heavily treated marrows with 0% CFU-GM recovery. Three patients of this group had their marrows treated with doses of Asta Z 7557 greater than the dose expected to reduce CFU-GM to undetectable levels. The control series (n = 13) consisted of nontreated marrows from the same patients.

Marrows of the three series and of the control were studied in continuous liquid cultures, over an irradiated allogeneic adherent layer, which did not give rise to CFU-GM per se, in control experiments. The level of CFU-GM regeneration was assessed every week, and results were expressed as a percentage of CFU-GM recovery per flask, evaluated from the amount of CFU-GM detected at time of initiation of the culture in the same but untreated marrow. The maximum level of CFU-GM regeneration was observed mostly by day 14 (days 7 through 21); medians were 132% (range 50% to 452%) in series 1; 36% (range 5% to 132%) in series 2; 8% (range 2% to 25%) in series 3 (Fig 2). CFU-GM maximum regeneration level, independent of time, was directly related to the level of residual CFU-GM after Asta Z 7557 in the marrow initially put in culture ($r = .804$, $P < .01$ by Spearman rank correlation test). None of the marrows in series 3 reached a 30% regeneration level, whereas 75% of marrows of series 1 and 2 did ($P < .01$, χ test). Untreated control marrows in the same conditions produced a median peak recovery of 76% (46% to 540%) earlier than in treated marrow—mostly by day 7.

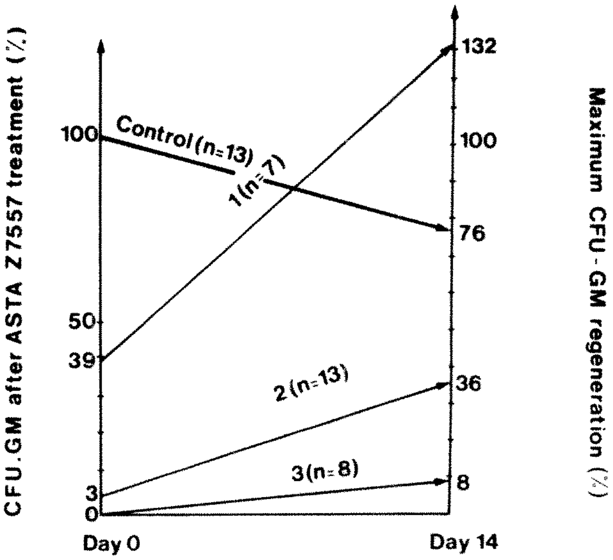

Fig 2. Continuous liquid cultures after treatment of bone marrow with Asta Z 7557: series of marrows treated with moderation (1), according to our protocol (2), or heavily (3), with median colony-forming unit granulocyte-macrophage (CFU-GM) recoveries in the input of 39%, 3%, and 0%, respectively.

Clinical Results

Engraftment. All patients, both in group 1 and group 2, engrafted consistently on leukocytes so that no reinfusion of untreated back-up marrow was necessary. The mean day for recovery of WBCs to 10^9/L for all patients was day 28.5 ± 9.4 (median day 30; range day 11 through 47) (Fig 3). It was significantly shorter for patients with ALL (mean day 20.5 ± 6, median day 19, range day 11 through 30) than for

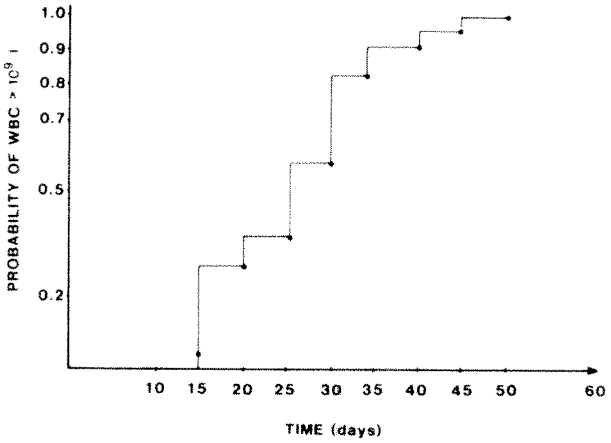

Fig 3. Recovery of leukocytes after autologous bone marrow transplantation with marrow cleansed by Asta Z 7557.

patients with ANLL (mean day 33 ± 7.8, median day 34, range day 19 through 47) ($P < .01$). In addition, recovery beyond the 10^9 leukocytes per Liter threshold was faster in patients with ALL. All eight patients receiving <100 CFU-GM/kg (including four patients whose marrows did not contain any detectable CFU-GM) engrafted well.

Of the ten evaluable patients in group 1, only four recovered a platelet count $>50.10^9$/L. Because most patients of this group had recurrent leukemia and died early, no conclusion can be drawn. In group 2, on the other hand, platelet recovery could be analyzed; in patients with ALL, median recovery to 50.10^9/L occurred on day 67 (range day 23 through 90). In contrast, in the eight patients with ANLL, one recovered on day 63, 5 recovered with delays >5 months, and 2 had not recovered at time of death, 3 and 16 months after ABMT. Three patients received platelet support for more than one year. Therefore, on the whole, recovery of hemopoiesis appeared to be faster in patients with ALL than in those with ANLL.

Toxicity. The major toxicity was hepatic. Two patients (UPNs 7 and 31), one in each group, developed liver venoocclusive disease (VOD) which contributed to and/or led to death two and three months after transplant. When compared with others, these two patients had additional hepatic risk factors prior to ABMT (additional ARA-C and VP-16 in the pretransplant regimen for both and presence of the Australia antigen for one).

Another patient (UPN 28) developed primary biliary cirrhosis that persisted for 11 months after ABMT and resolved spontaneously. Three additional patients (UPN 9, 26, and 27) developed a hepatitis episode of unknown origin which resolved spontaneously. In one case (UPN 9), liver biopsy showed alterations identical to those of acute graft-*v*-host disease (GVHD).

Bacterial sepsis was documented during aplasia in 18 patients. One patient died with disseminated aspergillosis and cytomegalovirus (CMV) infection (UPN 3). Viral infection was documented in 5 patients (2 CMV, 2 herpes simplex virus [HSV] infection, 1 herpes zoster) and was suspected in 5 others. Among other toxicities, we observed one severe but reversible wasting syndrome, with the presence of an IgG monoclonal spike (UPN 8).

Antileukemic efficacy and survival. Results were poor in group 1; the two patients still in CR or disease-free at time of writing have a short follow-up (nine and ten months). Three patients died with no evidence of leukemia, one of VOD on day 60, another of CMV and *Aspergillus* pneumonitis on day 100, and the third of severe gastrointestinal bleeding on day 330 while receiving steroids because of persistent thrombocytopenia, autoimmune manifestations, and a liver biopsy consistent with acute GVHD. Five patients relapsed early, within six months after transplant. Particularly disappointing was the course of patient 2 with a secondary leukemia following breast cancer, who not only had a leukemic relapse, but who also developed concomitantly recurrent breast tumor with multiple disseminated skin metastases.

In contrast, results in group 2 were more favorable: one patient with a M5 AML (UPN 32) and one patient with ALL (UPN 22) relapsed 6 and 15 months, respectively, after ABMT. Two patients died while disease-free 3 and 16 months after transplant, the first one of VOD and the other of viral infection. The nine other patients have remained in persisting CR, with a median follow-up of 22 months (8 to 27 months). Eight patients have remained in CR for more than 1 year (13+, 14+, 17+, 22+, 26+, 26+, 26+, and 27+ months). The median survival from initial diagnosis is 28 months.

The disease-free survival is 27% at nine months in group 1, as contrasted with 72% at two years in group 2 ($P < .01$) (Fig 4).

DISCUSSION

Renewed interest in the use of ABMT for the treatment of AL has recently occurred with the development of techniques to cleanse the marrow of residual tumor cells, using such different approaches as CV derivatives,[5,6,16-18] other drugs,[19] monoclonal antibodies,[20-22] immunotoxins,[23-25] or combinations of these.[26,27] We selected Asta Z 7557 (INN Mafosfamide, Cis 4 sulfoethylthiocyclophosphamide), a relatively stable CY derivative, which, after dissolution, undergoes a rapid spontaneous hydrolysis with liberation of 4 hydroperoxycyclophosphamide (4 HC). Asta Z 7557 and 4 HC are cytotoxic to many animal and human tumor cells, including L 1210 mouse leukemia with a reported 5 log cell kill,[28] the rat DMBA-induced[29] and BNML leukemias,[17] and the human Namalwa, CA 46, and BJAB 113 Burkitt's lymphoma cell lines with a reported 2 to 3.5 log clonogenic cell elimination.[26] In addition, in humans, CY is clinically active in ALL and has remained the standard drug for conditioning patients with both ALL and AML BM to transplantation.[30,31]

Our preliminary in vitro studies demonstrated an increased sensitivity of CFU-L to Asta Z over BFU-e, but not over CFU-GM, confirming our previous observations with 4 HC.[6] However, they proved to us that sensitivity to Asta Z was dependent on technical parameters such as RBC contamination. For this reason, and because in our hands the dose-response curve when using pure mononuclear fractions from Ficoll-Hypaque gradient-separated marrows was too

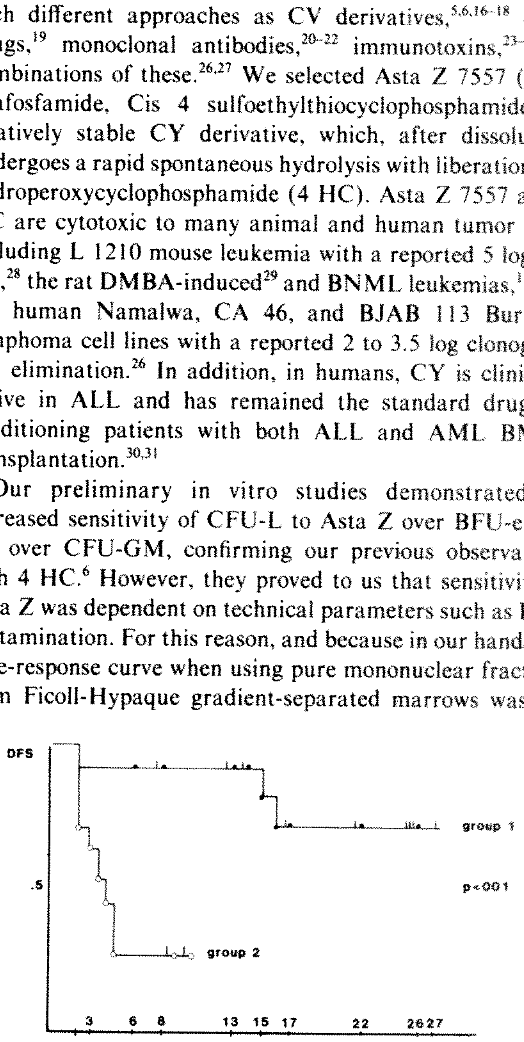

Fig 4. Disease-free survival after autologous bone marrow transplantation with marrow cleansed by Asta Z 7557 for acute leukemia in remission. Group 1, patients with advanced disease and/or poor prognosis criteria (O); group 2, patients in first complete remission and standard risk factors (●). Vertical marks (I) indicate patients at risk.

steep to select appropriate doses of the drug to work with, we elected to have all of our in vitro tests on buffy coat constantly adjusted to a final hematocrit of 5%. Similar observations have been made by others, and the inverse relation between Asta Z cytotoxicity to progenitor cells and RBC contamination responsible for nonspecific binding and diversion of the drug from the primary target is now well established.[18]

Our preliminary studies also showed a wide range of sensitivity from patient to patient; furthermore, a few patients tested sequentially (data not shown) revealed an increasing resistance to the drug that developed in parallel with progression of the disease. When we started the clinical trial, these observations prompted us to study the sensitivity to Asta Z in each individual patient immediately before marrow collection in order to select the dose for incubation rather than to use a constant dosage—hence, we used the PIT. As in our preliminary studies, the PIT showed considerable variations from patient to patient in response to identical drug concentrations, suggesting that an adjustment of the dose on an individual basis might be appropriate.

Because we could not directly evaluate the tumor log cell kill and because the biological importance of cells detected in the CFU-L assay is unknown, we decided to define the dose of Asta Z with which to incubate the marrow as the highest possible dose that would spare enough normal stem cells to ensure consistent engraftment. A residual amount of 5% ± 5% CFU-GM in the cleansed marrow was considered a safe margin beyond which further cytotoxicity would not be measurable, at least by conventional laboratory means. With this definition, doses of Asta Z ranging from 50 μg to 140 $\mu g/2.10^7$ buffy coat cells were necessary. For patients in first remission (group 2), the PIT predicted cytotoxicity with accuracy since the CFU-GM surviving fractions in the cleansed marrows ranged from 0% to 8%. On the other hand, in group 1, four discrepancies were noted (CFU-GM surviving fractions from 21% to 47%). Two of these occurred in our first two patients, and all four could be retrospectively attributed to technical errors. Since we initiated our program, other investigators have reported similar individual variations in sensitivity to Asta Z 7557 both in animal progenitor cell populations[17] and in human leukemic and lymphoid cells.[32] A heterogeneity in the DNA repair among WBCs has been postulated to be responsible for this variable sensitivity to alkylating agents.[32]

In mice, liquid cultures of highly concentrated spleen colony-forming unit (CFUs) suspensions produce an increase in CFU-GM, whereas stem-cell–depleted suspensions do not, which suggests that an increase in colony formation in liquid culture is a reflection of the proliferation capability of the cell suspension.[33] Our long-term culture studies indicated that even marrows totally depleted of CFU-GM still retained a self-regenerating ability. Although they confirmed the higher resistance of early progenitors to CY derivatives as observed in the BN rat model[17-34] and suggested by initial preliminary reports of successful autologous engraftment in humans with marrow deprived of CFU-GM,[35] these studies also showed that the self-renewal capacity was proportional to the surviving fraction of CFU-GM in the sample initially placed in culture, which reassured us in our intention not to go below the threshold of 5% residual CFU-GM in the cleansed marrow. All patients in our study engrafted, although receiving as predicted very low numbers of CFU-GM. The kinetics of recovery of peripheral blood cells were delayed, however. Recovery of leukocytes to $10^9/L$ occurred within a time considered usual[36,37] but further increase of WBC count beyond this level remained unusually slow. When considering group 2, in which the absence of detectable recurrent leukemia for the time of follow-up makes the data easier to analyze, platelet recovery appeared strikingly delayed, essentially in patients with ANLL, three of whom had to be supported with platelet transfusions for more than one year after ABMT. Whether the slower kinetics in ANLL reflect an effect of the underlying disease per se remains to be studied. On the whole, these observations suggest that the reinfused marrows were heavily treated, and that their engraftment potential was somewhat altered. Therefore, despite the absence of a clonogenic assay to deduce the tumor cell log reduction, it is likely that the marrows of our patients were indeed cleansed at the maximum tolerable rate.

In patients with poor-risk factors (group 1), our results were modest, since leukemia recurred in most patients. Whether these poor results should be attributed mainly to the selection of high-risk patients or to insufficient cleansing of the marrow, or both, cannot now be decided. In contrast, the results were better in standard-risk patients (group 2) since 9 of 13 patients are still in CR without maintenance chemotherapy, 8 to 27+ months after ABMT (median 22+ months), and 11 to 56+ months after initial diagnosis (median 28+ months). Eight patients have been off therapy for >1 year (4 ALL, 4 ANLL). Of the 4 patients autografted after long delays (>6 months), 2 died (1 after relapse) and 2 are long-term disease-free survivors. Therefore, it is unlikely that the long delay prior to graft in these patients has had any favorable incidence on the disease-free survival rate (72% at 2 years). Some centers have addressed the question of ABMT with cleansed marrow in second or subsequent remission: most relevant to our study is the trial of Yeager and co-workers, consisting of two phases using ABMT with marrow cleansed by 4 HC.[38] Compilation of the data from phases 1 and 2 indicated poor results in ALL (<10% disease-free survival at 3 years) and much better results in ANLL (disease-free survival of 40% at 3 years). Although very few centers have started a consolidation program with ABMT in first remission, reports from the European Bone Marrow Transplantation Group indicated a disease-free survival with a possible plateau beyond one year >50% both in ALL and ANLL, whether or not an attempt was made to cleanse the marrow.[4]

Although our results are only preliminary, and the follow-up is still too short, we believe that we can draw some conclusions from our study. First, Asta Z can be used to cleanse the marrow of patients with leukemia. Because there is a wide range of sensitivity to Asta Z 7557 from patient to patient, we consider that an individual adjustment of the dose is an option, the clinical relevance of which must be further tested. Second, ABMT with marrow cleansed by Asta Z brings a moderate benefit to patients with poor-risk leukemias. Patients with standard risk factors, on the other hand, may derive more marked improvement in terms of unmaintained disease-free survival. However, the follow-up is too short, and we cannot yet compare our results to those obtained with conventional chemotherapy or allogeneic BM transplantation. In addition, in the absence of randomized trials comparing the use of cleansed and noncleansed marrows, there is no way to evaluate the potential benefit of the cleansing procedure we selected. Despite these restrictions, we believe that our data support the view that ABMT with cleansed marrow in remission may become an alternative therapy for AL, at least for those patients who have no compatible donor for allogeneic transplantation, and that this view therefore deserves further investigation.

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REFERENCES

1. Bortin MM, Gale RP, Kay HEM, Rimm AA: Bone marrow transplantation for acute myelogenous leukemia. JAMA 249:1166, 1983

2. Thomas ED, Buckner CD, Clift RA, Fefer A, Johnson EL, Neuman PE, Sale GE, Sanders JE, Singer JW, Shulman H, Storb R, Weiden PL: Marrow transplantation for acute nonlymphoblastic leukemia in first remission. N Engl J Med 301:597, 1979

3. Barrett AJ, Kendra JR, Lucas CF, Joss DV, Joshi R, Desai M, Hugh Jones K, Phillips RH, Rogers TR, Tabara Z, Williamson S, Hobbs J: Bone marrow transplantation for acute lymphoblastic leukaemia. Br J Haematol 52:181, 1982

4. Gorin NC, Aegerter P, Parlier Y: for the working party on autologous bone marrow transplantation of the European Bone Marrow Transplantation Group: Autologous bone marrow transplantation for acute leukemia in remission: Second European survey. Exp Hematol 13:18, 1985 (suppl 17)

5. Sharkis SJ, Santos GW, Colvin M: Elimination of acute myelogenous leukemic cells from marrow and tumor suspensions in the rat with 4 hydroperoxycyclophosphamide. Blood 55:521, 1980

6. Douay L, Gorin NC, Gerota I, Najman A, Duhamel G: In vitro treatment of leukemic bone marrow for autologous transplantation. Exp Hematol 10:113, 1982 (suppl 12)

7. Chang LJA, Till JE, McCulloch EA: Cellular basis of self renewal in culture by human acute myelobastic leukaemia blast cell progenitors. J Cell Physiol 102:217, 1980

8. Pike BL, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76:77, 1970

9. Iscove NN, Sieber F: Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. Exp Hematol 3:32, 1975

10. Dexter RM, Allen TD, Lajtha LG: Conditions controlling the proliferation of hemopoietic stem cells in vitro. J Cell Physiol 91:335, 1977

11. Gorin NC, Douay L, David R, Stachowiak J, Parlier Y, Oppenheimer M, Najman A, Duhamel G: Delayed kinetics of recovery of hematopoiesis, following autologous bone marrow transplantation. The role of excessively rapid marrow freezing rates after the release of fusion heat. Eur J Cancer 19:485,1983

12. Finney DJ: Statistical Method in Biological Assay, 2nd ed. London, Griffin, 1971, p 452, 468

13. Winer BJ: Statistical Principles in Experimental Design, 2nd ed. Kogakusha, Tokyo, McGraw-Hill, 1971, p 167

14. Siegel S: Nonparametric Statistics for the Behavioral Sciences. New York, McGraw-Hill, 1956, p 75; 126; 202

15. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457, 1958

16. Korbling M, Hess AD, Tutschka PJ, Kaizer H, Colvin MO, Santos GW: 4 Hydroperoxycyclophosphamide: A model for eliminating residual human tumor cells and T lymphocytes from the bone marrow graft. Br J Haematol 52:89, 1982

17. Hagenbeek A, Martens ACM: Toxicity of Asta Z 7557 (INN Mafosfamide) to normal and leukemic stem cells: Implications for autologous bone marrow transplantation. Invest New Drugs 2:237, 1984

18. Herve P, Cahn JY, Plouvier E, Flesh M, Tamayo E, Leconte Des Floris R, Peters A: Autologous bone marrow transplantation for acute leukemia using transplant chemopurified with metabolite of oxazophorines (Asta Z 7557, INN Mafosfamide). First clinical results. Invest New Drugs 2:245, 1984

19. Stiff PJ, Wustrow TPU, Koester AR, Derisi MF, Clarkson B: In vivo and in vitro pharmacologic purification of bone marrow contaminated with tumor cells using VP 16-213 and nitrogen mustard, in Lowenberg B, Hagenbeek A (eds): Minimal Residual Disease in Acute Leukemia. Boston, Martinus Nijfoff, 1984, p 183

20. Bast RC, Ritz J, Lipton JM, Feeney M, Sallan SC, Nathan DG, Schlossman SF: Elimination of leukemic cells from human bone marrow using monoclonal antibody and complement. Cancer Res 43:1389, 1983

21. Lebacq-Verheyden AM, Ravoet AM, Bazin H, Sutherland DR, Tidman N, Greaves MF: Rat AL2, AL3, AL4 and AL5 monoclonal antibodies bind to the common acute lymphoblastic leukaemia antigen (CALLA gp 100). Int J Cancer 1983

22. Ramsay N, Lebien T, Nesbit M, McGlave P, Weisdorf D, Hurd D, Kenyon P, Goldman A, Kim T, Kersey J: Autologous bone marrow transplantation for acute lymphoblastic leukemia following marrow treatment with BA-1, BA-2, BA-3 and rabbit complement. Blood 66:508, 1985

23. Krolick KA, Uhr JW, Vitetta ES: Selective killing of leukaemia cells by antibody-toxin conjugates: Implications for autologous bone marrow transplantation. Nature 295:604, 1982

24. Kersey J, Vallera D, Ramsay N, Filipovich L, Stong R, Lebien T, Youle R, Neville D: Monoclonal antibodies for purging bone marrow of acute lymphoblastic leukemia or graft versus host producing cells: Antibody plus complement or immunotoxins, in Minimal Residual Disease in Acute Leukemia. Lowenberg B, Hagenbek A (eds): Boston, Martinus Nijhoff, 1984, p 233

25. Gorin NC, Douay L, Laporte JP, Lopez M, Zittoun R, Rio B, David R, Stachowiak J, Jansen J, Cazellas P, Poncelet P, Liance MC, Voisin GA, Salmon Ch, Le Blanc G, Deloux J, Najman A, Duhamel G: Autologous bone marrow transplantation with marrow decontaminated by immunotoxin T 101 in the treatment of leukaemia and lymphoma. First clinical observations. Cancer Treat Rep 69:953, 1985

26. De Fabritiis P, Bregni M, Lipton J, Greenberger J, Nadler L, Rothstein L, Korbling M, Ritz J, Bast RC Jr: Elimination of clonogenic Burkitt's lymphoma cells from human bone marrow using 4 hydroperoxycyclophosphamide in combination with monoclonal antibodies and complement. Blood 65:1064, 1985

27. Uckun F, Houston L, Ramakrishnan S, Hunstein W: In vitro

tumor cell purging by immunotoxins in combination with 4 hydroperoxycyclophosphamide. Exp Hematol 12, 1974 (abstr 172)

28. Zaharko DS, Covey JM, Horpel G: Observations on the effects of cyclophosphamide, phosphoramide mustard, and some activated oxazaphosphorines on murine L1210 leukemia. Invest New Drugs 2:149, 1984

29. Zeller WJ, Berger MR, Matys R, Schuhmacher J: Antineoplastic activity of Asta Z 7557 (INN Mafosfamide) in transplanted and autochthonous experimental rodent tumors. Investigational N Drugs 2:175, 1984

30. Santos GW, Sensenbrenner LL, Burke PJ: Allogeneic marrow grafts in man using cyclophosphamide. Transplant Proc 6:345, 1974

31. Thomas ED, Buckner CD, Banaji M: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation and allogeneic marrow transplantation. Blood 49:511, 1977

32. Osieka R, Pannenbacker P, Gottfried Schmidt C: Macromolecular DNA-damage in murine and human leukemic and lymphoid cells after in vitro exposure to Asta Z 7557. Invest New Drugs 2:161, 1984

33. Nicola NA, Metcalf D, von Melchner H, Burgess AW: Isolation of murine fetal hemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood 58:376, 1976

34. Hagenbeek A, Martens ACM: Autologous bone marrow transplantation in acute leukemia: Separation of hematopoietic stem cells from clonogenic leukemia cells by 4 hydroperoxycyclophosphamide. Exp Hematol 10:14, 1982 (suppl 11) (abstr 23)

35. Kaizer H, Stuart RK, Brookmeyer R, Beschorner WE, Braine HG, Burns WH, Fuller DJ, Korbling M, Mangan KF, Saral R, Sensenbrenner L, Shadduck RK, Shende AC, Tutschka PJ, Yeager AM, Zinkham WH, Colvin OM, Santos GW: Autologous bone marrow transplantation in acute leukemia: A phase 1 study of in vitro treatment of marrow with 4 hydroperoxycyclophosphamide to purge marrow cells. Blood 65:1504, 1985

36. Gorin NC, David R, Stachowiak J, Salmon Ch, Petit JC, Parlier Y, Najman A, Duhamel G: High-dose chemotherapy and autologous bone marrow transplantation in acute leukemias, malignant lymphomas and solid tumors. A study of 23 patients. Eur J Cancer 17:557, 1981

37. Herzig GP: Autologous marrow transplantation in cancer therapy. Prog Hematol 12:1, 1981

38. Yeager AM, Kaizer H, Braine HG, Colvin M, Rowley S, Saval R, Santos GW: Autologous bone marrow transplantation (ABMT) in acute leukemia: Phase 1 and 2 studies of ex vivo marrow treatment with 4 hydroperoxycyclophosphamide (4HC). Int J Cell Cloning 3:237, 1985

Quinidine Purpura: Evidence That Glycoprotein V Is a Target Platelet Antigen

By Raphael B. Stricker and Marc A. Shuman

Quinidine-induced thrombocytopenia has been associated with both immune complex and autoantibody binding to platelets. In the present study, serum antibody from six of six patients with quindine purpura was shown by immunoblotting to bind to a single platelet membrane protein of mol wt 80,000. This target protein was absent from Bernard-Soulier (BSS) platelets. $F(ab)_2$ prepared from one patient's serum also bound to this protein, indicating autoantibody rather than immune complex binding to the target antigen. Antibody binding to the 80-kd protein was preserved after treatment of platelets with concentrations of trypsin or chymotrypsin that completely removed glycoprotein Ib (GPIb). Preincubation of platelet proteins with one patient's serum blocked binding of a polyclonal rabbit antibody against glycoprotein V (GPV), indicating that these antibodies recognize the same antigen. By wheat germ affinity chromatography, GPV was shown to copurify with GPIb. Quinidine-induced antibody bound to the wheat germ-purified GPV but not to GPIb. We conclude that quinidine purpura is associated with autoantibody directed against platelet GPV.

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QUINIDINE PURPURA is an immune-mediated thrombocytopenia induced by quinidine or its metabolites.[1] Although previous studies have postulated an immune complex mechanism of platelet destruction in this disease,[1,2] more recent work indicates that autoantibody binding to platelets may be the causative mechanism.[3,4] The target platelet antigen in quinidine purpura has been reported to be glycoprotein Ib (GPIb), based on immunoprecipitation studies in one patient[5] and binding inhibition studies using alloantibody against GPIb to block platelet lysis by drug-dependent antibody in another patient.[6] However, a direct relationship between GPIb and quinidine-dependent antibody has not been established.[1]

We previously used immunoblotting with patient serum to define target platelet antigens in homosexual men with immune thrombocytopenic purpura[7] and in a patient with acquired Bernard-Soulier syndrome (BSS).[8] In the present study, we used immunoblotting of platelet proteins to determine the nature of target platelet antigen(s) in quinidine-induced thrombocytopenia.

MATERIALS AND METHODS

Serum samples and $F(ab)_2$ preparation. Blood was obtained from six patients with quinidine-induced thrombocytopenia. All patients were taking quinidine sulfate, and all were thrombocytopenic at the time that blood was drawn. The platelet counts returned to normal in all six patients following discontinuation of the drug. Three of the patient samples had serum quinidine levels of 0.6, 0.7, and 1.7 mg/dL, whereas the other three samples had undetectable levels (<0.2 mg/dL) at the time that blood was drawn. Sera from three patients with thrombocytopenia due to phenytoin or trimethoprim/sulfamethoxazole and from six quinidine-treated patients with normal platelet counts were used as controls. The quinidine-treated controls had serum drug levels ranging from 0.9 to 5.1 mg/dL. Drug-induced thrombocytopenia was documented by an indirect platelet antibody assay showing binding of serum antibody to control platelets in the presence of the specific drug.[9] Rabbit antisera against GPV[10] and GPIb were generously provided by Dr David R. Phillips and Dr Joan Fox of the Gladstone Institute Foundation, San Francisco. $F(ab)_2$ fragments were prepared as previously described[7] from the staphylococcal protein A-purified IgG fraction of blood from one patient with quinidine-induced thrombocytopenia. The IgG was used at a concentration of 2 mg/mL and the $F(ab)_2$ was used at a concentration of 3 mg/mL for immunoblotting.

Platelet and GPV preparation. Control and BSS platelets were isolated as previously described.[7] The BSS platelets[11] were generously provided by Dr Margaret Johnson of the Medical Center of Delaware, Newark. Trypsin-treated platelets were prepared according to the method of Phillips.[12] Chymotrypsin-treated platelets were prepared by the method of Greenberg et al[13] using 10 U/mL of α-chymotrypsin (Sigma, St Louis). GPV was partially purified and concentrated by a modification of the method of Berndt and Phillips[10]: freshly prepared platelets (4×10^9/mL) were incubated with elution buffer (0.01 mol/L of Hepes, 1 mmol/L of EDTA, 0.3 mol/L of NaCl, ph 7.6) at 37 °C for 24 hours. The resultant platelet eluate was dialyzed and concentrated overnight at 4 °C against phosphate-buffered saline (PBS) containing 5 mmol/L of EDTA and 10% polyethylene glycol. The eluate was then suspended in PBS-EDTA at a protein concentration of 1.1 mg/mL. Protein measurements were made by a modified Lowry technique.[14] The GPV preparation was then solubilized in 2% sodium dodecyl sulfate (SDS) sample buffer. It was subsequently shown to be free of GPIb by immunoblotting with rabbit anti-GPIb antibody (data not shown).

Wheat germ affinity chromatography was performed according to the method of Nachman et al.[15] Normal platelets (4×10^9/mL) were solubilized in 1% SDS. The SDS was then diluted to 0.05% in a solution containing 5 mmol/L of EDTA and 1% (vol/vol) aprotinin, and the solubilized platelets were layered over a column of wheat germ coupled to agarose (E-Y Labs, San Mateo, Calif). Elution was performed using 0.2 mol/L of *N*-acetylglucosamine. The nonadherent fraction (WGA I) and adherent *N*-acetylglucosamine-eluted fraction (WGA II) were dialyzed against PBS-EDTA-1% aprotinin and solubilized in 2% SDS at equivalent protein concentrations (3.5 mg/mL).

Immunoblotting. Fifty-microliter aliquots of platelet samples, partially purified GPV, WGA I, or WGA II, were electrophoresed in SDS-8% polyacrylamide gels or SDS-5% polyacrylamide gels under nonreducing conditions.[7] The samples were transferred to nitrocellulose and blotted as previously described.[7] Patient serum samples were diluted 1:100 in Tris-buffered saline (TBS) containing 1% gelatin. Rabbit serum was diluted 1:500 in the same buffer.

Blocking experiments were performed as follows: nitrocellulose strips were incubated with patient or control serum for one hour,

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followed by washing in TBS and incubation with rabbit anti-GPV for two hours. Binding of rabbit antibody to GPV was detected with biotin-conjugated goat $F(ab)_2$ anti-rabbit IgG (Tago, Burlingame, Calif) and avidin-peroxidase (Cappel, Malvern, Pa). The anti-rabbit antibody did not react with human IgG.

RESULTS

Figure 1 shows the results of immunoblotting using serum incubated with platelet proteins that have been transferred to nitrocellulose after electrophoresis in SDS-8% polyacrylamide gels. In contrast to control serum (lane A), antibody binding to a platelet protein with a mol wt of 80,000 was detected using patient serum (lane B). Identical results were obtained with all six sera from patients with quinidine-induced thrombocytopenia. Sera from the six quinidine-treated controls and from the three patients with other drug-induced thromobcytopenias showed no antibody binding to this antigen (data not shown). Binding did not occur when the patient's serum was incubated with BSS platelets (lane C), indicating absence of the target antigen on these platelets. Incubation of patient serum with partially purified GPV revealed binding to a protein of identical mol wt (lane D). $F(ab)_2$ (3 mg/mL) prepared from serum of the only patient tested also bound to this target protein (lane E). No antibody binding was seen in the region of GP IX (mol wt 22,000, not shown).

Figure 2 shows the results of immunoblotting with platelet proteins that have been transferred to nitrocellulose after electrophoresis in SDS-5% polyacrylamide gels. In lane A, patient serum again shows antibody binding to an 80-kd protein at the bottom of the gel. The band at 150 kd corresponds to IgG in the platelet preparation. This band is also detected when BSS platelets are incubated with patient serum, but the 80-kd band is not seen in these platelets (lane B). The 80-kd band corresponds to concentrated GPV, seen in lane C after incubation with patient serum. In lane D, incubation of platelet proteins with rabbit anti-GPIb yields a band at 170 kd. This band is not seen in lane A, indicating that the patient's antibody does not bind to GPIb.

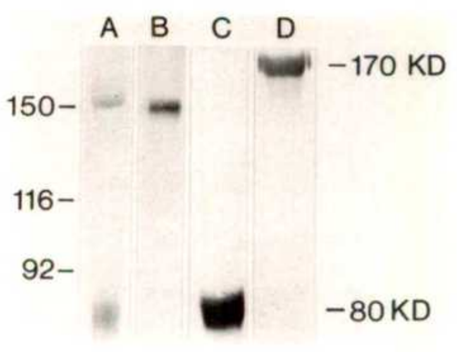

Fig 2. Comparison of antibody binding to glycoproteins V and Ib (GPV and GPIb). Platelet proteins or partially purified GPV were subjected to sodium dodecyl sulfate (SDS) 5% polyacryamide gel electrophoresis (PAGE), transferred to nitrocellulose, and incubated with patient serum or rabbit anti-GPIb antibody as follows: lane A, control platelets incubated with patient serum; lane B, Bernard-Soulier syndrome (BSS) platelets incubated with patient serum; lane C, partially purified GPV incubated with patient serum; lane D, control platelets incubated with anti-GPIb antibody. Note antibody binding to 80-kd protein in control platelets but not in BSS platelets and corresponding to GPV. Band at 150-kd in lanes A and B corresponds to IgG in platelet preparations. Serum antibody does not bind to GPIb (mol wt 170,000) in control platelets (lane A).

Figure 3 shows binding and blocking experiments using rabbit anti-GPV antibody. In contrast to control rabbit serum (lane A), the anti-GPV antiserum bound to an 80-kd protein detected in control platelets (lane B) and in the partially purified GPV preparation (lane C). In the blocking experiment, control platelet protein (lane E) or purified GPV

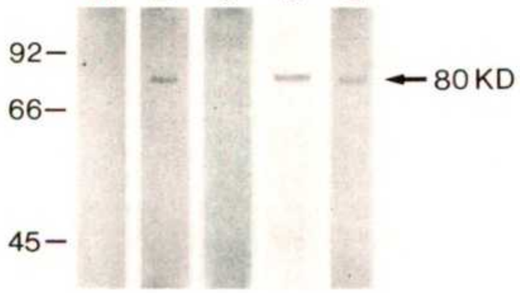

Fig 1. Identification of antibody against glycoprotein V (GPV) in patients with quinidine purpura. Solubilized platelet proteins were subjected to sodium dodecyl sulfate (SDS)-8% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and incubated in stepwise fashion with test serum or $F(ab)_2$, biotin-conjugated goat $F(ab)_2$ anti-human Ig, and avidin-peroxidase. Lane A, control platelets incubated with control serum; lane B, control platelets incubated with patient serum; lane C, Bernard-Soulier syndrome (BSS) platelets incubated with patient serum; lane D, purified GPV incubated with patient serum; lane E, control platelets incubated with patient $F(ab)_2$ (3 mg/mL). Note binding of patient antibody and $F(ab)_2$ to 80-kd control platelet protein and purified GPV, but not to BSS platelets (arrow).

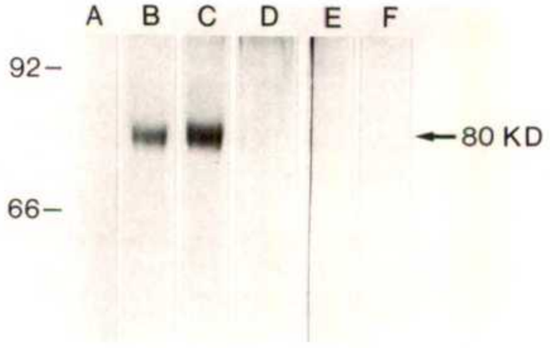

Fig 3. Binding of rabbit antiglycoprotein V (GPV) on immunoblots. Lane A, platelets incubated with control rabbit serum; lane B, platelets incubated with anti-GPV antibody; lane C, purified GPV incubated with anti-GPV antibody; lane D, Bernard-Soulier syndrome (BSS) platelets incubated with anti-GPV antibody; lane E, platelets incubated with patient serum followed by anti-GPV antibody; lane F, purified GPV incubated with patient serum followed by anti-GPV. Rabbit antibody binding was detected using biotin-conjugated goat antirabbit IgG. Note anti-GPV antibody binding to 80-kd protein (arrow) that is blocked by preincubation with patient serum in lanes E and F.

(lane F) was first incubated with patient serum, followed by rabbit anti-GPV. Binding of the rabbit antiserum was abolished by preincubation with patient serum, indicating that both antibodies recognize the same binding site. Preincubation with either normal human serum or serum from a patient with phenytoin-induced thrombocytopenia failed to block binding of the rabbit antibody to GPV (data not shown).

Figure 4 shows binding of patient serum and rabbit anti-GPV to platelet proteins eluted from a wheat germ affinity column. The patient's serum and anti-GPV antibody bound to an 80-kd antigen in the WGA II (adherent) fraction (lanes B and D) but not in the WGA I (nonadherent) fraction (lanes A and C). The WGA II fraction also contained a 170-kd protein that reacted with rabbit anti-GPIb (data not shown). Thus, GPV copurifies with GPIb when both are subjected to wheat germ affinity chromatography. Binding of rabbit antibody to GPV was blocked by preincubation with patient serum (lane E), again showing that both antibodies bind to GPV. Preincubation with control human serum did not block rabbit antibody binding to GPV (data not shown). For comparison, binding of patient IgG (2 mg/mL) to GPV in platelets is shown in lane F.

Figure 5 shows the results of immunoblotting using trypsin-treated and chymotrypsin-treated platelets. In contrast to control platelets (lane A), rabbit anti-GPIb antibody failed to bind to trypsin-treated platelets (lane B) or to chymotrypsin-treated platelets (lane C). However, patient serum still bound to GPV in both of these platelet preparations (lanes E and F), indicating that GPV is not removed by exposure to trypsin or chymotrypsin. The band at 150 kd in lanes D through F again corresponds to IgG in the platelet preparations. Thus, enzyme-treated platelets that are deficient in GPIb retain the ability to bind anti-GPV antibody.

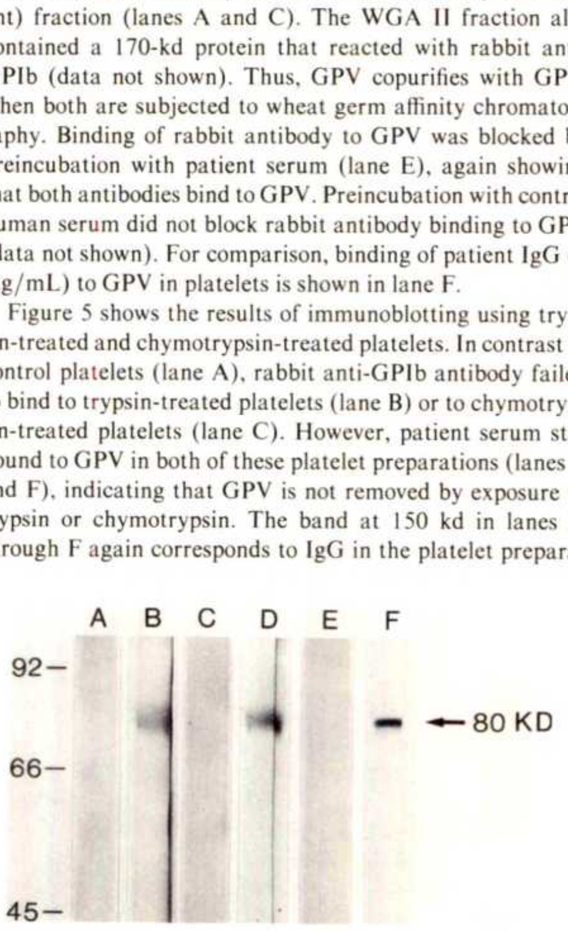

Fig 4. Immunoblot of antibody binding to wheat germ-purified platelet glycoproteins. Control platelets were solubilized and subjected to wheat germ affinity chromatography. The nonadherent (WGA I) and adherent (WGA II) platelet glycoprotein fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and incubated with patient serum or rabbit anti-GPV antibody. Lane A, WGA I incubated with patient serum; lane B, WGA II incubated with patient serum; lane C, WGA I incubated with anti-GPV antibody; lane D, WGA II incubated with anti-GPV antibody; lane E, WGA II incubated with patient serum followed by rabbit anti-GPV antibody and biotin-conjugated goat anti-rabbit IgG; lane F, control platelets incubated with patient IgG (2 mg/mL). Note binding to 80-kd glycoprotein in WGA II fraction that corresponds to GPV in platelet preparation (arrow). Binding of rabbit anti-GPV antibody to WGA II is blocked by preincubation with patient serum (lane E).

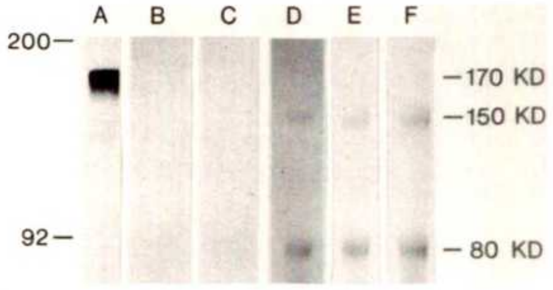

Fig 5. Immunoblot of platelets after exposure to trypsin or chymotrypsin. Control platelets were treated with trypsin or chymotrypsin. The platelet proteins were then subjected to sodium dodecyl sulfate (SDS) 5% polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose, and incubated with rabbit anti-glycoprotein Ib (GPIb) or patient serum. Anti-GPIb was incubated with untreated platelets (lane A), trypsin-treated platelets (lane B), or chymotrypsin-treated platelets (lane C). Patient serum was incubated with untreated platelets (lane D), trypsin-treated platelets (lane D), or chymotrypsin-treated platelets (lane F). Note that rabbit antibody binding to GPIb (mol wt 170,000) is abolished by trypsin or chymotrypsin treatment, but patient antibody binding to glycoprotein V (GPV) (mol wt 80,000) is retained. Bands at 150,000 daltons in lanes D through F correspond to IgG in platelet preparations detected by anti-human immunoglobulin.

DISCUSSION

Although quinidine-induced thrombocytopenia is a well-described entity, the target platelet antigen and the mechanism of platelet destruction in this disease remain controversial. Previous reports have suggested that a drug-antibody immune complex binds to the platelet F(c) receptor, resulting in destruction of platelets as "innocent bystanders."[1,2] However, recent findings are inconsistent with the innocent bystander hypothesis. Kunicki et al[6] showed that binding of heat-aggregated human IgG to the F(c) receptor on platelets failed to inhibit binding of quinidine-dependent antibody. Conversely, formalin fixation of platelets under conditions that do not inhibit binding of immune complexes abolished binding of drug-induced antibody.[1] Furthermore, Christie et al[3] demonstrated that anti-F(ab)$_2$ antibody inhibited binding of quinidine-dependent antibody to platelets, suggesting the presence of autoantibody rather than immune complex binding to the target antigen. Similar observations were made by Smith et al[4] using a different technique.

Initial studies showed that BSS platelets failed to bind antibody from patients with quinidine purpura,[1] suggesting that the binding site is GPIb, known to be deficient in these platelets. In addition, Degos et al[16] demonstrated immunoprecipitation of radiolabeled GPIb by an IgG alloantibody isolated from the serum of a BSS patient. This alloantibody blocked binding of quinidine-dependent antibody to normal platelets in the only patient studied,[6] further implicating GPIb as the antibody binding site. Because GPIb was reported to be an immune complex receptor on platelets,[17] it was postulated that the mechanism of platelet destruction in quinidine purpura involved binding of drug–antibody complex to this protein. However, when platelets were treated with concentrations of trypsin or chymotrypsin that completely removed GPIb, they retained 80% of the receptor activity for quinidine-dependent antibody.[1] Based on this observation, Kunicki et al suggested that a membrane protein other than GPIb may be responsible for drug-induced antibody binding in quinidine purpura.[1]

Our results suggest that GPV is a target platelet antigen recognized by quinidine-associated antibody. GPV has three characteristics that have been attributed to the receptor for this antibody. First, the glycoprotein is missing from BSS platelets,[18] explaining the absence of antibody binding to these platelets. Second, we have found that removal of GPIb using trypsin or chymotrypsin fails to remove GPV from normal platelets. These GPIb-deficient platelets retain the ability to bind quinidine-induced anti-GPV antibody. Third, our results using wheat germ affinity chromatography demonstrate for the first time that GPV copurifies with GPIb in the wheat germ-adherent fraction. Kunicki et al previously showed that 80% of antigenic activity for quinidine-dependent antibody can be isolated in the wheat germ-adherent platelet membrane fraction.[6] Our findings suggest that at least in the patients we have studied, this antigenic activity is due to the presence of GPV rather than GPIb in the adherent fraction. However, we have not excluded the possibility that quinidine induces antibody formation against GPIb as well as GPV.

Previous studies have shown that quinidine-dependent antibody binds to whole platelets[1,3,4] or to Triton-solubilized platelet membranes[5,6] only in the presence of quinidine. Our results using platelet membranes fixed in nitrocellulose show that binding of the anti-GPV antibody occurs with serum, purified IgG, and $F(ab)_2$ in the apparent absence of the drug. Addition of exogenous quinidine (1 mmol/L final concentration) to the incubation mixture did not increase antibody binding to GPV on the nitrocellulose membranes. A possible explanation for antibody binding in the absence of drug is that platelet membrane fixation in nitrocellulose may render the target antigen more accessible to the antibody, even in the absence of quinidine. An attractive hypothesis based on our findings is that quinidine causes platelet destruction in two steps: (1) it induces autoantibody production against GPV, and (b) it directly interacts with the platelet membrane to make GPV more accessible to the antibody. Although antibody binding to intact platelets or to soluble membranes requires both steps, binding to membranes fixed in nitrocellulose does not require the presence of drug. This hypothesis is also consistent with the "neoantigen" model proposed by Christie et al[3]: The putative neoantigen corresponds to GPV exposed by quinidine. Further testing of purified antibody and soluble GPV in the presence or absence of the drug may resolve this issue.

Another possibility is that quinidine purpura involves binding of both drug-dependent and non–drug-dependent antibodies to platelets, as suggested in two recent reports.[19,20] Drug-dependent antibody binding may not be detectable by the immunoblot technique. Whether non–drug-dependent anti-GPV antibody plays a role in the pathogenesis of thrombocytopenia is unclear. We believe that this antibody is significant, however, since it was present in all six patients with quinidine purpura but in none of the six quinidine-treated patients with normal platelet counts. The relationship of the anti-GPV antibody to the development of thrombocytopenia must still be determined.

Our results are consistent with $F(ab)_2$ binding to a target platelet antigen in at least one patient. How quinidine induces autoantibody formation against platelets must still be determined. We have found serum antibody against GPV in two patients with acute leukemia refractory de novo to platelet transfusions, as well as in two children with ITP (R.B. Stricker and M.A. Shuman, unpublished observations, 1985). In addition, Beardsley et al[21] recently demonstrated antibodies against GPV in three children with varicella-associated thrombocytopenia. Thus, antibody production against GPV may result from an alteration in immune surveillance induced by a specific drug or underlying disease. This type of immune dysfunction has been invoked to explain the lupuslike syndrome associated with quinidine and other drugs.[22,23] The mechanism of autoantibody induction by quinidine requires further study.

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REFERENCES

1. Kunicki TJ, Christie DJ, Aster RH: The human platelet receptor(s) for quinine/quinidine-dependent antibodies. Blood Cells 9:293, 1983

2. Christie DJ, Aster RH: Drug-antibody-platelet interaction in quinine- and quinidine-induced thrombocytopenia. J Clin Invest 70:989, 1982

3. Christie DJ, Mullen PC, Aster RH: Fab-mediated binding of drug-dependent antibodies to platelets in quinidine- and quinine-induced thromobcytopenia. J Clin Invest 75:310, 1985

4. Smith ME, Jordan JV, Reid DM, Jones CE, Shulman NA: Drug-antibody binding to platelets is mediated by the Fab domain and is not Fc-dependent. Blood 64:91a, 1984 (abstr)

5. Chong BH, Berndt MC, Koutts J, Castaldi PA: Quinidine-induced thrombocytopenia and leukopenia: Demonstration and characterization of distinct antiplatelet and antileukocyte antibodies. Blood 62:1218, 1983

6. Kunicki TJ, Russell N, Nurden AT, Aster RH, Caen JP: Further studies of the human platelet receptor for quinine- and quinidine-dependent antibodies. J Immunol 126:398, 1981

7. Stricker RB, Abrams DI, Corash L, Shuman MA: Target platelet antigen in homosexual men with immune thrombocytopenia. N Engl J Med 313:1375, 1985

8. Stricker RB, Wong D, Saks SR, Corash L, Shuman MA: Acquired Bernard-Soulier syndrome. J Clin Invest 76:1274, 1985

9. Corash L, Rheinschmidt M: Detection of platelet antibodies using a fluorescence-activated flow cytometric technique, in Fahey

JL, Friedman H, Rose N, (eds): Manual of Clinical Laboratory Immunology, 3rd ed (in press)

10. Berndt MC, Phillips DR: Purification and preliminary physicochemical characterization of human platelet membrane glycoprotein V. J Biol Chem 256:59, 1981

11. Walsh PN, Mills DCB, Pareti FI, Stewart GJ, MacFarlane DE, Johnson MM, Egan JJ: Hereditary giant platelet syndrome. Br J Haematol 29:639, 1975

12. Phillips DR: Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. Biochemistry 11:4582, 1972

13. Greenberg JP, Packham MA, Guccione MA, Harfenist EJ, Orr JL, Kinlough-Rathbone RL, Perry DW, Mustard JF: The effect of pretreatment of human or rabbit platelets with chymotrypsin on their responses to human fibrinogen and aggregating agents. Blood 54:753, 1979

14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

15. Nachman RL, Tarasov E, Weksler RB, Ferris B: Wheat germ agglutinin affinity chromatography of human platelet membrane glycoproteins. Thromb Res 12:91, 1977

16. Degos L, Tobelem G, Lethielleux P, Levy-Toledano S, Caen J, Colombani J: Molecular defect in platelets from patients with Bernard-Soulier syndrome. Blood 50:899, 1977

17. Moore A, Ross GD, Nachman RL: Interaction of platelet membrane receptors with von Willebrand factor, ristocetin, and the Fc region of immunoglobulin G. J Clin Invest 62:1053, 1978

18. Berndt MC, Gregory C, Chong BH, Zola H, Castaldi PA: Additional glycoprotein defects in Bernard-Soulier syndrome: Confirmation of genetic basis by parental analysis. Blood 62:800, 1983

19. Garty M, Ilfeld D, Kelton JG: Correlation of a quinidine-induced platelet-specific antibody with development of thrombocytopenia. Am J Med 79:253, 1985

20. Lerner W, Caruso R, Faig D, Karpatkin S: Drug-dependent and non–drug-dependent antiplatelet antibody in drug-induced immunologic thrombocytopenic purpura. Blood 66:306, 1985

21. Beardsley DS, Ho JS, Beyer EC: Varicella-associated thrombocytopenia: Antibodies against an 85-kd thrombin-sensitive protein (GPV?). Blood 66:286a, 1985 (abstr)

22. Lavie CJ, Biundo J, Quinet RJ, Waxman J: Systemic lupus erythematosus (SLE) induced by quinidine. Arch Intern Med 145:446, 1985

23. Shoenfeld Y, Schwartz RS: Immunologic and genetic factors in autoimmune disease. N Engl J Med 311:1019, 1984

Juvenile Chronic Myelogenous Leukemia: Characterization of the Disease Using Cell Cultures

By Zeev Estrov, Tom Grunberger, Helen S.L. Chan, and Melvin H. Freedman

To characterize juvenile chronic myelogenous leukemia (JCML), the proliferative properties of bone marrow (BM) and peripheral blood (PB) cells from nine patients were studied using assays for CFU-C and CFU-GEMM and liquid cultures. All specimens showed two reproducible abnormalities: impaired growth of normal hematopoietic progenitors and excessive proliferation of monocyte-macrophage colonies in the absence of exogenous colony-stimulating activity (CSA). Cytogenetic studies in one patient indicated that the CFU-C were malignant because BM cells at diagnosis and monocyte-macrophage colonies showed an abnormal karyotype, whereas PB lymphocytes did not. In contrast to JCML, PB from six adults with Philadelphia (Ph^1) chromosome-positive chronic myelogenous leukemia (Ph^1 + CML) yielded CSA-dependent CFU-C colonies which were composed of granulocytes, macrophages, or both, as well as exuberant growth of BFU-E colonies. Co-cultures of JCML BM adherent or nonadherent cells with normal BM resulted in suppression of normal hematopoietic colony formation. Supernatant from JCML adherent cells in liquid culture or plasma from newly diagnosed untreated JCML patients also suppressed control BM colony growth in a dose-dependent manner. These findings confirm that JCML is a malignant disorder of monocytic lineage and suggest that the mechanism of hematopoietic failure in JCML is mediated by an inhibitory monokine secreted by malignant JCML cells.

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JUVENILE CHRONIC myelogenous leukemia (JCML) is a severe hematopoietic malignancy of early childhood characterized by an acute course with a median survival of less than ten months. When compared to Philadelphia (Ph^1) chromosome-positive chronic myelogenous leukemia (Ph^1 + CML), there are marked clinical, hematological, and cytogenetic differences, and JCML should be considered as a separate entity.[1,2]

Because the disorder is uncommon, clinical and investigative experience with JCML has been limited. Recently, we diagnosed nine patients with JCML, a relatively large number for a single institution. To characterize the disease and understand its pathogenesis, we studied bone marrow (BM) and peripheral blood (PB) cells from the patients using a panel of in vitro techniques. Our data show that JCML is a malignant proliferation of monocytic lineage and implicate a monokine humoral inhibitor for the marrow failure of normal hematopoietic elements that characterizes this condition.

MATERIALS AND METHODS

Subjects. All nine patients fit the classical clinical and laboratory description of JCML.[1,2] They had varying combinations of lymphadenopathy, skin manifestations, variable splenomegaly, pallor, and hemorrhagic signs. Most had anemia, leukocytosis, monocytosis, thrombocytopenia, and nucleated RBCs in PB (Table 1). BM aspirates were cellular, with granulocytic hyperplasia, monocytosis, active erythropoiesis, and reduced to absent megakaryocytes. The patients were followed for eight months to six years.

For control studies, BM was obtained from hematologically normal patients in whom an aspiration was performed as part of a medical investigation or from healthy adult volunteers. PB was also obtained from normal volunteers and from adult patients with classical Ph^1+ CML. These studies were performed with informed consent and were approved by the Human Experimentation Committee of our institution.

Preparation of cells. Heparinized BM or PB cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 g, 4 °C) for 20 minutes to remove neutrophils and RBCs.

Adherent cell fractionation. After Ficoll-Paque, BM mononuclear cells were incubated in plastic Petri dishes or flasks (Falcon Plastics, Becton Dickinson, Oxnard, Calif) with 10% fetal calf serum (FCS) in α-medium for 90 minutes at 37 °C. In this modification of the technique described by Shaw et al,[3] nonadherent cells were removed by gentle pipetting and repeated vigorous washing of plates with α-medium. The procedure was repeated until no more cells adhered to the Petri dishes. Nonadherent cells harvested in this way contained $<3\%$ monocytes as confirmed by the following techniques: microscopic differential count of up to 500 cells prepared with Wright's stain; nonspecific (α-naphthyl butyrate) esterase staining[4]; and immunocytochemical analysis[5] using anti-MY-4 monoclonal antibody (Coulter Immunology, Hialeah, Fla) to identify monocyte-promonocyte cells. In the co-culture studies, normal BM mononuclear nonadherent cells were layered over 1 to 2×10^5 cells that were adherent to 35-mm Petri dishes.

Liquid culture assay. Adherent PB mononuclear cells from JCML patients, two patients at diagnosis and one patient during remission, were incubated in 25-cm² Falcon flasks with 20% FCS in α-medium at 37 °C, with 5% CO_2 in air in a humidified atmosphere. For each study, an appropriate control was performed using PB mononuclear cells (prepared in an identical manner) from five normal donors. After seven days, the supernatants were harvested, frozen at -80 °C, and stored for testing in co-culture studies with control BM.

CFU-C assay. The CFU-C assay was performed as previously described.[6] In brief, nucleated BM cells (10^5) or PB cells (5×10^5) were cultured in 0.8% methylcellulose, 30% FCS, 20% colony-stimulating activity (CSA), which was prepared from PB leukocytes of a normal volunteer[7] and α-medium. The culture mixture was placed in 35-mm Petri dishes in duplicate and maintained at 37 °C, with 5% CO_2 in air in a humidified atmosphere. Colonies were counted after 14 days using an inverted microscope. A CFU-C colony was defined as a cluster of ≥ 20 cells consisting of granulo-

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Table 1. Clinical and Laboratory Data on Nine Patients With JCML at Diagnosis When In Vitro Studies Were Performed

Patient	1	2	3	4	5	6	7	8	9
Sex	M	F	F	M	F	F	F	M	M
Age (mo)	5	30	3	5	30	60	18	12	51
Hemoglobin (g/dL)	9.3	5.4	7.9	5.7	5.6	9.1	4.9	7.7	10.7
WBC (× 10^9/L)	76	960	69.9	11.5	96	15	36.8	36.4	49.5
Platelets (× 10^9/L)	143	125	372	10	46	24	51	53	60
PB monocytes (%)	19	1	20	12	13	6	7	41	7
PB blasts (%)	2	23	9	1	3	0	1	0	8
BM M-E ratio	10.5:1	7:1	1:1	1.5:1	1:1	1:2	5:1	1:1	5:1
BM monocytes (%)	1	15	15	12	25	7	10	5	10
BM blasts (%)	2	20	3	3	1	1	16	0	5

JCML, juvenile chronic myelogenous leukemia; PB, peripheral blood; BM, bone marrow.

cytes, monocyte-macrophages, or both. Microscopically, pure granulocytic colonies could be easily distinguished from pure macrophage colonies because of smaller cells and tighter clustering in the former. Individual colonies were plucked from the cultures with a micropipette and spread on glass slides, and the cellular composition was determined microscopically using Wright's stain and nonspecific (α-naphthyl butyrate) esterase staining with and without treatment with fluoride.[4]

CFU-GEMM assay. The CFU-GEMM assay was performed according to Fauser and Messner.[8,9] In brief, 2×10^5 nucleated BM or PB cells were cultured in methylcellulose with Iscove's modified Dulbecco's medium (Ontario Cancer Institute, Toronto), 30% AB serum or plasma obtained from a pool of five normal donors, 5% leukocyte-conditioned medium prepared with phytohemagglutinin (PHA-LCM),[10] human erythropoietin 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver) or sheep erythropoietin 2.0 U/mL (Connaught Laboratories, Willowdale, Ontario), and 5×10^5 mol/L of 2-mercaptoethanol. One milliliter of the culture mixture was placed in 35-mm Petri dishes and incubated at 37 °C with 5% CO_2 in air in a humidified atmosphere. All cultures were evaluated after 14 days for the number of BFU-E colonies, defined as an aggregate of >500 hemoglobinized cells or three or more erythroid subcolonies, CFU-C colonies of granulocytic or macrophage cells or both, and mixed colonies containing all elements. Individual colonies were plucked from the cultures with a micropipette and analyzed for cellular composition.

Cytogenetic analysis. Cytogenetic studies on fresh BM cells were done by the direct method of Hozier and Lindquist.[11] For the study of CFU-C chromosomes, colcemid (0.1 μg/mL) was added for one hour to the culture plates on day 14 of incubation and was then removed by washing with phosphate-buffered saline (PBS). Individual CFU-C colonies were plucked from the culture dishes and pooled, and chromosome analysis was performed by routine methods.[12] Banding of chromosomes was done using the trypsin-Giemsa technique.[13]

Statistical analysis. The probability of significant differences between colony numbers was determined by Student's *t* test.

RESULTS

As shown in Table 2, when either PB or BM cells from JCML patients were cultured in the CFU-C assay, the specimens yielded an exceptionally high number of colonies with a plating efficiency of three- to > 20-fold higher than that of controls ($P < .001$). Although colonies from PB and BM from four of seven JCML patients declined when CSA was omitted from the cultures, similar to results with those of controls, the excessive plating efficiency persisted. In three of seven JCML patients, no decline in colony growth was seen when CSA was omitted. Individual JCML colonies at harvest showed all stages of the monocytic lineage, including blast forms, promonocytes, monocytes, and histiocytic macrophages, whereas in controls at least 50% of the colonies were granulocytic. These findings were confirmed by morphological, cytochemical, and immunocytochemical analysis of individual colonies plucked from the cultures and by examination of the cellular composition of entire harvested culture plates.

BM from patient 7 (Table 1) had an abnormal karyotype. In 100% BM cells in metaphase, there were two distal deletions from the long arms of chromosomes 1 and 13 and additional terminal bands on 5q and 12q; the PB lymphocyte karyotype was normal (46 XX). In individual JCML colonies plucked from cultures of PB or BM, the abnormal karyotype was seen in 87% and 100% of the cells in metaphase, respectively. During the patient's clinical and laboratory remission, cytogenetic studies performed on PB,

Table 2. PB and BM Cultures From Patients With JCML Using the CFU-C Assay

	Patient No.	PB (CFU-C/5 × 10^5 Cells Plated)	BM (CFU-C/10^5 Cells Plated)
With CSA	1	385 ± 19	228 ± 21
	2	444 ± 14	661 ± 17
	3	>1,000	>1,000
	4	185 ± 14	261 ± 13
	5	>1,000	>1,000
	6	187 ± 11	321 ± 22
	7	>1,000	>1,000
	8 Controls	51 ± 9	56 ± 8
Without CSA	1	133 ± 9	98 ± 15
	2	274 ± 17	151 ± 12
	3	>1,000	>1,000
	4	107 ± 13	112 ± 10
	5	>1,000	>1,000
	6	93 ± 8	123 ± 11
	7	>1,000	>1,000
	8 Controls	23 ± 7	28 ± 9

PB, peripheral blood; BM, bone marrow; JCML, juvenile chronic myelogenous leukemia; CSA, colony-stimulating activity.

Data are mean ± SD.

BM, and CFU-C revealed a normal karyotype, and CFU-C colony growth was normal in number and dependent on exogenous CSA.

PB colony growth from JCML patients, six adults with Ph^1+ CML, and controls is shown in Table 3. All studies were performed on samples depleted of adherent cells and in the presence or absence of added CSA. JCML PB yielded a two- to > 14-fold increase in colonies as compared with those of controls ($P < .0001$). In sharp contrast to those of controls, JCML colony numbers from five of six patients did not decline when CSA was omitted from the cultures. PB from patients with Ph^1+ CML yielded colony numbers higher than those of controls but much lower than those of JCML patients, and the colonies declined 50% when CSA was omitted. Cellular composition of JCML colonies was exclusively monocyte-macrophage, whereas in Ph^1+ CML and in controls, it consisted of varying ratios of both granulocyte and monocyte-macrophage cells.

PB from JCML and Ph^1+ CML patients was cultured in the CFU-GEMM assay (Table 4). All of the studies were performed on samples depleted of adherent cells and in the presence or absence of PHA–LCM. JCML PB gave rise to excessive growth of monocyte-macrophage colonies that was unchanged when PHA–LCM was omitted from the cultures. No BFU-E or mixed colonies were seen in any cultures of JCML PB whether PHA–LCM was present or not. Ph^1+ CML PB also yielded increased CFU-C numbers as compared with those of controls ($P < .001$), but the colonies were mainly granulocytic, and they declined ~40% when PHA–LCM was omitted. In comparison to JCML PB, Ph^1+ CML PB gave rise to unusually high numbers of granulocytic CFU-C and BFU-E colonies. The BFU-E growth was particularly exuberant and persisted in the absence of PHA–LCM, in contrast to those of controls.

During clinical and laboratory remission, patients 7 and 8 were studied again. PB samples from both yielded normal colony numbers per culture (85 and 58 CFU-C colonies, 50 and 82 BFU-E colonies, 5 and 3 mixed colonies, respectively).

BM from two JCML patients was depleted of adherent cells and co-cultured in varying ratios with control BM that was similarly prepared (Fig 1). With increasing numbers of JCML cells in co-culture, an increase in monocyte-macrophage colony formation was observed, concurrent with a decline of granulocytic CFU-C and BFU-E growth. With co-cultures containing 50% JCML BM cells, BFU-E growth was abolished, and granulocytic CFU-C growth was markedly suppressed.

Table 3. Comparison of PB Cell Cultures From JCML and Ph^1 + CML Patients Using the CFU-C Assay

	CFU-C/5 × 10^5 Cells Plated	
Patients*	With CSA	Without CSA
Pt 1	178 ± 6	192 ± 13
Pt 2	551 ± 11	380 ± 13
Pt 3	>1,000	>1,000
Pt 4	156 ± 3	152 ± 4
Pt 5	>1,000	>1,000
Pt 6	159 ± 2	162 ± 8
6 Pts with Ph^1 + CML	110 ± 33 (G:M = 3:2)	51 ± 28 (G:M = 3:2)
12 Controls	70 ± 29 (G:M ~1)	5 ± 3 (G:M ~1)

PB, peripheral blood; JMCL, juvenile chronic myelogenous leukemia; Ph^1+ CML, Philadelphia chromosome in addition to chronic myelogenous leukemia; CSA, colony-stimulating activity; M, macrophage; G, granulocyte.

Adherent cell fraction was removed from all specimens prior to plating. The data are presented as mean ± SD.

*See Table 1 for data.

As seen in Fig 2, adherent JCML BM cells from two patients were left in Petri dishes, and control BM cells were cultured over them in a CFU-GEMM assay. In these cultures, BFU-E, CFU-C, and mixed-colony formation was significantly inhibited as compared with results in studies of control BM cells cultured over control adherent cells ($P < .01$).

The supernatant harvested from the liquid cultures of JCML PB adherent cells from two patients was tested for its effect on control marrow colony growth (Fig 3). With increasing concentrations of both JCML supernatants, there was a dose-dependent suppression of CFU-C, BFU-E, and mixed-colony formation as compared with supernatant obtained from flasks of control PB adherent cells that had no effect on cell growth. Supernatant from one of these patients tested during remission yielded results similar to controls.

Figure 4 shows the results of experiments in which various ratios of JCML plasma-control AB plasma were prepared and then substituted in the cultures for control AB plasma that is routinely used in the CFU-GEMM assay. The effect of these preparations was tested on control marrow colony growth. The results from both patients were identical. With increasing percentages of JCML plasma in the cultures, there was a dose-dependent decline in CFU-C and BFU-E

Table 4. PB Cultures From JCML and Ph^1 + CML Patients in the CFU-GEMM Assay

	Colonies/2 × 10^5 Cells Plated					
	With PHA–LCM			Without PHA–LCM		
Diagnosis	CFU-C	Mixed	BFU-E	CFU-C	Mixed	BFU-E
Pt 7	>1,000	0	0	>1,000	0	0
Pt 8	189 ± 8	0	0	152 ± 2	0	0
Pt 9	137 ± 9	0	0	123 ± 5	0	0
5 Pts with Ph^1+ CML	146 ± 83 (G > M)	8 ± 5	314 ± 153	84 ± 20 (G > M)	0	169 ± 108
5 Controls	72 ± 16 (G:M = 1)	2 ± 1	130 ± 13	37 ± 17 (G:M = 1)	0	12 ± 7

Adherent cell fraction was removed from all specimens prior to plating. The data are presented as mean ± SD.

PB, peripheral blood; JCML, juvenile chronic myelogenous leukemia; Ph^1+ CML, Philadelphia chromosome positive in addition to CML; PHA–LCM, phytohemagglutinin–leukocyte-conditioned medium; M, macrophage; G, granulocyte.

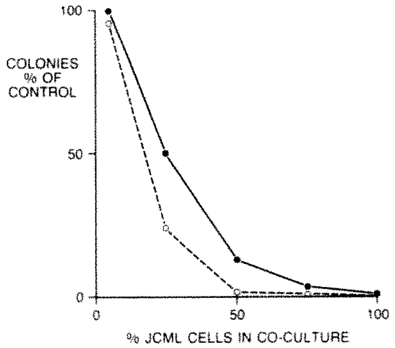

Fig 1. Co-cultures of juvenile chronic myelogenous leukemia bone marrow (JCML BM) in varying ratios with control BM in the CFU-GEMM assay. Adherent cells were depleted from all BM specimens prior to culture. Data from two patients with JCML are presented as percentages of control colony numbers. (The mean numbers of control colonies were 125 CFU-C and 130 BFU-E per 10^5 cells plated.) ●, Granulocyte CFU-C; ○, BFU-E.

colony numbers. When 90% JCML plasma was substituted for AB plasma, colony growth was almost completely abolished. This inhibitory effect was not detected when remission plasma was tested.

DISCUSSION

There have been only a few research reports on JCML,[14-17] and these have been contradictory. Altman et al were the first to claim that juvenile "chronic granulocytic" leukemia is a misnomer, and that the disease is really a panmyelopathy with prominent monocytic involvement and circulating monocyte colony-forming cells.[14,15] Others disagreed because large numbers of granulocytic CFU-C were demonstrated in cultures of JCML BM and PB.[16,17] Our data are clear-cut and resolve this issue. Our major finding in cell cultures of JCML BM and PB was an excessively high number of CFU-C colony numbers in both the CFU-C and the CFU-GEMM culture assay systems. The cellular composition of these colonies was exclusively monocyte-macrophage, as proven by their microscopic appearance in the culture plates, cytospin preparations of harvested plates, morphology, cytochemistry, and surface markers of individual colonies plucked from cultures.

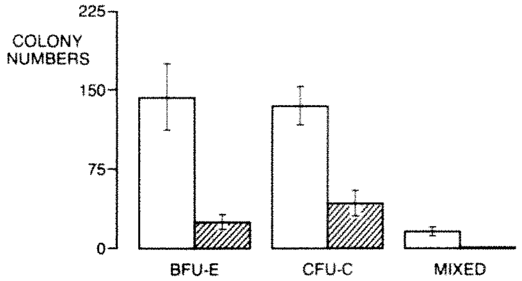

Fig 2. Co-cultures of juvenile chronic myelogenous leukemia (JCML) adherent cells with control bone marrow (BM) in the CFU-GEMM assay. Adherent cells were left in Petri dishes, and control BM cells were cultured over them. Data from two patients with JCML are presented as mean colony numbers. □, Control adherent cells; ▨, JCML adherent cells.

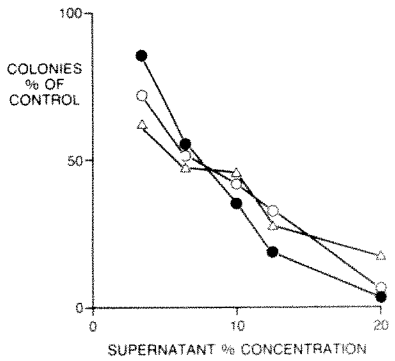

Fig 3. The effect of supernatant harvested from juvenile chronic myelogenous leukemia peripheral blood (JCML PB) adherent cells grown in liquid culture on control BM colony growth in the CFU-GEMM assay. The concentration of the supernatant is expressed as percentage of total volume per culture. (The mean numbers of control colonies were 150 CFU-C, 135 BFU-E, and 15 mixed colonies per 10^5 cells plated.) △, CFU-C; ○, BFU-E; ●, mixed.

The abnormally high plating efficiency of these monocyte colony-forming cells is most unusual because differential counts of JCML BM and PB prior to culture show granulocytic hyperplasia and only a mild to moderate monocytosis. As shown in Table 1, BM is not "replaced" by monocytic elements as in acute leukemia. Clearly, cells of monocytic lineage in JCML have a unique growth advantage in vitro that is a hallmark of the disease. Because most CFU-C from PB of patients with Ph^1+ CML were of granulocytic lineage, confirming other reports,[15,18,19] the in vitro characteristics of JCML were markedly different and emphasize that this condition is a separate entity.

We also found atypical growth requirements for JCML CFU-C. For proliferation of normal BM or PB CFU-C into colonies in vitro, a humoral growth factor is essential and can be provided either by adding exogenous CSA or by leaving intact the adherent cell fraction which produces endogenous

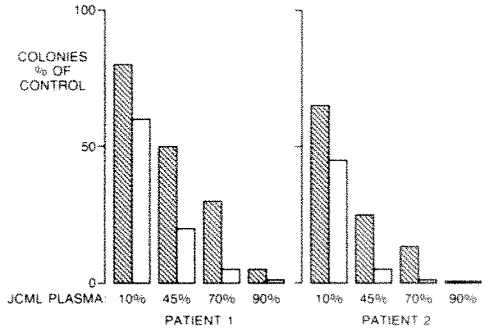

Fig 4. The effect of juvenile chronic myelogenous leukemia (JCML) plasma on control marrow colony growth in the CFU-GEMM assay. Various ratios of JCML plasma-control AB plasma were substituted in culture for control AB plasma. JCML plasma volumes are expressed as percentages of total plasma in culture. Bars represent percentages of the number of colonies grown with control AB plasma. (The mean numbers of control colonies were 145 CFU-C and 135 BFU-E per 10^5 cells plated.) ▨, CFU-C; □, BFU-E.

CSA.[20] In our studies using CFU-GEMM and CFU-C assays, much of the CFU-C growth from patients with Ph^1+ CML and from controls was CSA-dependent, because the colony numbers declined significantly when both adherent cells and CSA were omitted from the cultures. In sharp contrast, added CSA was not essential for JCML CFU-C to proliferate into colonies.

The cytogenetic findings in patient 7 provided good evidence that the JCML monocyte-macrophage CFU-C were involved in the malignant process. The abnormal karyotype was demonstrated in fresh and in cultured BM cells but not in PB lymphocytes. Moreover, when the patient attained a chemotherapy-induced remission, the karyotype could no longer be found, and the BM and PB CFU-C and CFU-GEMM growth characteristics became normal. These results are convincing indications that JCML is a malignant proliferation of monocytic lineage. However, we cannot be certain that the disease is confined exclusively to this lineage.

The CFU-GEMM assay has not been used before to study JCML; we found that it provided new information. Unlike the CFU-C assay, it profiles other hematopoietic lineages and gives more of an overview of cellular events. The CFU-GEMM assay confirmed the high numbers of monocyte-macrophage colony-forming cells. Moreover, JCML CFU-C colonies developed in the absence of an exogenous humoral growth stimulus in this assay, as well as when adherent cells were depleted from the cultured samples. There were also abnormalities of erythropoiesis and mixed-colony formation using standard plating conditions. No detectable BFU-E or mixed colonies were identified in JCML PB, a result clearly different than was found with PB from Ph^1+ CML patients and controls. In addition, the hematopoietic growth pattern in vitro became normal during remission, as noted in patients 7 and 8. To assess whether the CFU-GEMM assay has applications as a diagnostic tool or as a monitor of chemotherapy efficacy in JCML, further studies using a wide range of cell concentrations appear to be indicated.

When data shown in Tables 2, 3, and 4 are reviewed, one may ask why normal hematopoietic colony growth did not increase after removal of the inhibitory adherent cell fraction. One explanation is that all pathologic inhibitory cells may not have been adherent. A second explanation is that by depleting the adherent cell layer, we probably removed normal elements that produce CSA and other humoral stimuli, as well as the inhibitory cells. Thus, the net effect was unchanged, and the inhibitory activity prevailed.

In an attempt to explain the growth advantage in vitro of JCML monocyte-macrophage cells and the suppression of BFU-E and granulocytic CFU-C, we studied the interaction of JCML adherent cell fractions and the supernatant from cultures of these fractions. It was clear that the presence of either the adherent cells themselves or the medium in which they were cultured was extremely inhibitory to control BM colony formation, indicating that the JCML adherent cells, which are primarily monocyte-macrophage, produce and secrete a "monokine" that can suppress normal hematopoiesis in vitro. Moreover, by mixing nonadherent BM cells from JCML patients and from controls, we found the same inhibitory effect, suggesting that young undifferentiated JCML cells were capable of excreting the same inhibitory substance. This substance may be the "leukemia inhibitory activity" described by Broxmeyer et al[21,22]; however, JCML "monokine" has much more suppressive properties on colony growth. Because plasma from JCML patients also produced inhibition of control BM colony formation, confirming the report of Suda et al,[17] it is likely that the monokine has in vivo relevance and may be an important mechanism of production of the anemia and thrombocytopenia in this disorder.

REFERENCES

1. Altman AJ, Schwartz AD: Malignant Diseases of Infancy, Childhood and Adolescence. Philadelphia, Saunders, 1983, p 228

2. Nathan DG, Oski FA: Hematology of Infancy and Childhood. Philadelphia, Saunders, 1981, p 1009

3. Shaw GM, Levy PC, LoBuglio AF: Human monocyte antibody-dependent cell-mediated cytotoxicity to tumor cells. J Clin Invest 62:1172, 1978

4. Tucker SB, Pierre RV, Jordan RE: Rapid identification of monocytes in a mixed mononuclear cell preparation. J Immunol Methods 14:267, 1977

5. Li CY, Ziesmer SC, Yam LT, English MC, Janckila AJ: Practical immunochemical identification of human blood cells. Am J Clin Pathol 81:204, 1984

6. Iscove NN, Senn JS, Till JE, McCulloch EA: Colony formation by normal and leukemic human marrow cells in culture: Effect of conditioned medium from human leukocytes. Blood 37:1, 1971

7. Amato D, Freedman MH, Saunders EF: Granulopoiesis in severe congenital neutropenia. Blood 47:531, 1976

8. Fauser AA, Messner HA: Granuloerythropoietic colonies in human bone marrow, peripheral blood, and cord blood. Blood 52:1243, 1978

9. Messner HA, Fauser AA: Culture studies of human pluripotent hemopoietic progenitors. Blut 41:327, 1980

10. Aye MT, Niho Y, Till JE, McCulloch EA: Studies of leukemic cell populations in culture. Blood 44:205, 1974

11. Hozier JC, Lindquist LL: Banded karyotypes from bone marrow: A clinical useful approach. Hum Genet 53:205, 1980

12. Moorhead PS, Nowell PC, Hellman WJ, Battips DM, Hungerford DA: Chromosome preparations of leukocytes cultured from human peripheral blood. Exp Cell Res 20:613, 1960

13. Seabright M: A rapid banding technique for human chromosomes. Lancet 2:971, 1971

14. Altman AJ, Palmer CG, Baehner RL: Juvenile "chronic granulocytic" leukemia: A panmyelopathy with prominent monocytic involvement and circulating monocyte colony-forming cells. Blood 43:341, 1974

15. Altman AJ, Baehner RL: In vitro colony forming characteristics of chronic granulocytic leukemia in childhood. J Pediatr 86:221, 1975

16. Barak Y, Levin S, Vogel R, Cohen IJ, Wallach B, Nir E, Zaizov R: Juvenile and adult types of chronic granulocytic leukemia of childhood: Growth patterns and characteristics of granulocyte-macrophage colony forming cells. Am J Hematol 10:269, 1981

17. Suda T, Miura Y, Mizoguchi H, Ijima H, Eguchi M, Kaku H, Ide C: Characterization of hemopoietic precursor cells in juvenile-type chronic myelocytic leukemia. Leuk Res 6:43, 1982

18. Crist WM, Ragab A, Moreno H, Pereira F, Foster JC:

Granulopoiesis in chronic myeloproliferative disorders in children. Pediatrics 61:889, 1978

19. Chervenick PL, Ellis LD, Pan SF, Lawson AL: Human leukemic cells—In vitro growth of colonies containing Philadelphia (Ph^1) chromosome. Science 174:1134, 1971

20. Quesenberry P, Levitt L: Hematopoietic stem cells (second of three parts). N Engl J Med 301:819, 1979

21. Broxmeyer HE, Jacobsen N, Kurland J, Mendelsohn N, Moore MAS: In vitro suppression of normal and granulocyte stem cells by inhibitory activity derived from leukemia cells. J Natl Cancer Inst 60:497, 1978

22. Broxmeyer HE, Grossbard E, Jacobsen N, Moore MAS: Persistence of leukemia inhibitory activity during remission of acute leukemia. N Engl J Med 301:346, 1979

Activation of Human Neutrophils by Monoclonal Antibody PMN7C3: Cell Movement and Adhesion Can Be Triggered Independently From the Respiratory Burst

By David A. Melnick, Tova Meshulam, Aurelio Manto, and Harry L. Malech

Anti-neutrophil monoclonal antibody PMN7C3 (IgG_3) recognizes glycoproteins bearing the oligosaccharide lacto-*N*-fucopentaose III, including the C3bi receptor, LFA-1, and p150,95 on the plasma membrane and a group of granule-associated glycoproteins. We have previously shown that binding of this antibody to polymorphonuclear leukocytes (PMNs) stimulates a transient rise in cytosolic free calcium concentration but does not trigger the neutrophil respiratory burst. We now demonstrate that binding of PMN7C3 (and five other monoclonal antibodies recognizing the same antigen) to human neutrophils activates several other cellular responses. Addition of PMN7C3 to monolayers of neutrophils induces a rapid change in cell shape followed by pseudopod formation and increased migration. With incubation at 37 °C, the neutrophils aggregate in clusters (leukoagglutination). Quantitation of cell movement in a multiwell chemotaxis assembly or by migration of PMNs under agarose revealed that PMN7C3 is both chemotactic and chemokinetic. Pretreatment with the antibody inhibits subsequent chemotactic response to other stimuli. Monoclonal antibodies binding to other neutrophil antigens do not mimic these effects. These data suggest that cell movement and adhesion can be triggered independently from the respiratory burst. PMN7C3 may be a useful probe with which to study the events that link receptor-ligand binding to cellular response.

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POLYMORPHONUCLEAR leukocytes (PMNs) can be activated to migrate to sites of inflammation and to generate toxic mediators of the inflammatory response. The events that link receptor-ligand binding to cell activation remain uncertain.

PMN7C3 is an anti-human neutrophil murine monoclonal antibody that inhibits opsonized zymosan-induced respiratory burst but enhances phagocytosis.[1,2] It recognizes glycoproteins bearing the oligosaccharide lacto-*N*-fucopentaose III, including the C3bi receptor (CR3), LFA-1, and p150,95 on the plasma membrane and a group of granule-associated glycoproteins.[2] Binding of PMN7C3 results in capping of antigen–antibody complexes on the cell surface and induces a transient rise in free cytosolic calcium concentration, $(Ca^{2+})_i$, but does not trigger the respiratory burst.[1-3] Thus, a transient rise in $(Ca^{2+})_i$ cannot be a sufficient signal for activation of this response.

In the present study, we show that binding of PMN7C3 to human neutrophils stimulates shape changes, leukoagglutination, chemokinesis, and chemotaxis. These data suggest that activation pathways leading to cell movement and adhesion can be triggered independently from those leading to the respiratory burst.

MATERIALS AND METHODS

Cell preparation. Venous blood from human volunteers was mixed with heparin (10 U/mL). PMNs (>95% purity) were prepared by sedimentation of erythrocytes with 3% dextran followed by centrifugation of leukocytes over Ficoll-Hypaque gradients (Histopaque 1083, Sigma Chemical Co., St. Louis).[4] Residual erythrocytes were removed by hypotonic lysis. PMNs were suspended in ice-cold Hank's balanced salt solution (HBSS) until use.

Preparation of monoclonal antibodies. Murine hybridoma lines secreting anti-human neutrophil monoclonal antibodies were selected as previously described.[1,2] Five IgM antibodies (95G8, 43B9, 74D8, 66B7, and 80C9) bind to the same antigen as PMN7C3 (IgG_3) as determined by Western blotting pattern and competitive binding studies. Control antineutrophil monoclonal antibodies (31D8, 13F6, 41C5, 8C7, and 55F3) bind to other antigens. Monoclonal immunoglobulin was partially purified from malignant ascites by two successive precipitations with 45% ammonium sulfate. Antibodies were stored in phosphate-buffered saline (PBS) at −70 °C until thawed for use. Additional monoclonal antibodies were kindly provided by Dr Samuel Wright (IB4; anti-CR3) and Dr John O'Shea (3D9; anti-CR1). Hybridoma line M1/70, secreting anti-MAC-1, was obtained from the American Type Culture Collection.

Leukoagglutination assay. Leukoagglutination of adherent neutrophils was assayed by modification of the technique of Lalezari and colleagues.[5,6] PMNs (5×10^6/mL HBSS) were added to each of 24 wells on plastic tissue culture plates (Costar, Cambridge, Mass). After a ten-minute incubation at 37 °C, nonadherent cells were aspirated, leaving behind a homogeneous monolayer of neutrophils. The medium was then replaced with HBSS alone or buffer containing 3.3×10^{-7} mol/L of monoclonal antibody. In some experiments, cytochalasin B (5 µg/mL) was added five minutes prior to the addition of monoclonal antibody. The monolayers were then incubated for 60 minutes at 37 °C. After incubation, the monolayers were examined under phase optics and scored for leukoagglutination (0, homogeneous monolayer; 4+, all cells aggregated in clusters) by two observers blinded to the daily protocol (concordance > 95%). In some experiments (as indicated below), incubation was carried out at room temperature rather than at 37 °C.

Time-lapse videomicroscopy. The events that followed binding of monoclonal antibody to the surface of adherent neutrophils were recorded on time-lapse videotapes. Monolayers of PMN were prepared as above and observed on the temperature-controlled stage of an inverted phase microscope. Images were recorded at 100× magnification using a Panasonic video camera and time-lapse video cassette recorder with internal clock. These tapes were used to assess the time sequence of shape and motility changes which occurred after addition of monoclonal antibody or other stimuli to the incubation buffer.

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Chemotaxis and chemokinesis. Neutrophil migration was quantitated using a multiwell chemotaxis assembly (Neuro Probe, Bethesda, Md) modeled after that designed by Falk et al.[7] Potential chemoattractants (in gelatin veronal buffer) were loaded in the lower wells and separated from PMNs [in Gey's buffer with 2% bovine serum albumin (BSA)] by filters with 3-μm pores (Neuro Probe, Bethesda, Md). After incubation (30 minutes at 37 °C), the filters were fixed, stained, and examined at 40× magnification. The distance from the top of the filter to the last focal plane in which two neutrophils were clearly visualized was measured as an index of migration as originally described by Zigmond and Hirsch.[8] Random migration was defined as the distance migrated toward buffer without added chemoattractant. The distance migrated toward a potential chemoattractant was defined as directed migration. To differentiate between chemotactic and chemokinetic effects, a "checkerboard analysis" was carried out in which the compounds to be tested were added both above and below the filter to vary the gradient systematically.[8,9]

Chemotaxis was also assayed by migration of neutrophils on plastic plates under 1% agarose.[10,11] Plastic tissue culture dishes (60 mm) were coated with 0.5% gelatin which was then overlayed with 6 mL of 1% agarose in minimal essential medium (MEM) containing 20 mmol/L of HEPES, 10 mmol/L of $NaHCO_3$, and 0.5% gelatin. A metal dye was then used to punch a radial array of triplicate wells in the agarose layer. A potential chemoattractant was placed in the outer well, and buffer was placed in the inner well of each triplicate set; 2.5×10^5 PMNs were then added to the center well, and the plates were incubated at 37 °C for two or three hours. After incubation, plates were fixed by sequential additional of methanol and formaldehyde, and the agarose layer was removed. PMNs adherent to the plastic dish were then stained with Giemsa. The distance from the edge of the center well to the leading front of PMNs advancing toward chemoattractant (directed migration) and toward buffer (random migration) was measured at 40× magnification using a micrometer. In experiments in which monoclonal antibodies were used as potential chemoattractants, plates were incubated overnight prior to addition of PMNs to allow for gradient formation.

Inhibition of chemotaxis by monoclonal antibodies. Inhibition of chemotactic response by monoclonal antibodies was examined by pretreating neutrophils with varying concentrations of monoclonal immunoglobulin for 15 minutes at 4 °C, after which cells were washed with buffer to remove unbound antibody. Migration toward *N*-formyl norleucyl leucyl phenylalanine (fNLLP) or zymosan-activated serum (ZAS) was then measured by the techniques above. Migration by antibody-treated cells was compared to migration by control PMNs pretreated with buffer lacking monoclonal antibody.

Reagents. ZAS was prepared on the day of use by incubating pooled human serum with prehydrated zymosan (10 mg/mL) for 20 minutes at 37 °C. All reagents were purchased from Sigma except as indicated.

Human experimentation. All blood specimens were collected following guidelines approved by the Institutional Review Board for Human Experimentation of University Hospital.

RESULTS

PMN7C3 induces adherent neutrophils to leukoagglutinate. Lalezari and co-workers observed that naturally occurring anti-neutrophil antisera (derived from mothers of infants with isoimmune neonatal neutropenia) could induce adherent PMNs to migrate into large clusters in an active, energy-dependent process. He termed this response leukoagglutination.[5,6] We screened a panel of anti-neutrophil monoclonal antibodies for the ability to induce this response. Preformed PMN monolayers in multiwell plastic tissue culture plates were incubated with monoclonal antibody (3.3×10^{-7} mol/L) or buffer for 60 minutes at 37 °C. As shown in Fig 1, incubation with PMN7C3 (but not a control anti-neutrophil monoclonal IgG) induced the adherent neutrophils to migrate into clusters. This reaction did not occur if cells were incubated at room temperature rather than at 37 °C, suggesting that it is an active process. Furthermore, fixation of neutrophils with 1% paraformaldehyde prior to antibody treatment blocked leukoagglutination, although it does not inhibit binding of PMN7C3 as detected by enzyme-linked immunosorbent assay (ELISA).[1,2] In addition, leukoagglutination was blocked by pretreatment of neutrophils with cytochalasin B (5 μg/mL), suggesting that this response requires an intact cytoskeleton.

The results of leukoagglutination assays with a panel of anti-neutrophil monoclonal antibodies are summarized in Table 1. In addition to PMN7C3 (IgG_3), we have isolated five other monoclonal antibodies (all IgM) which bind to the

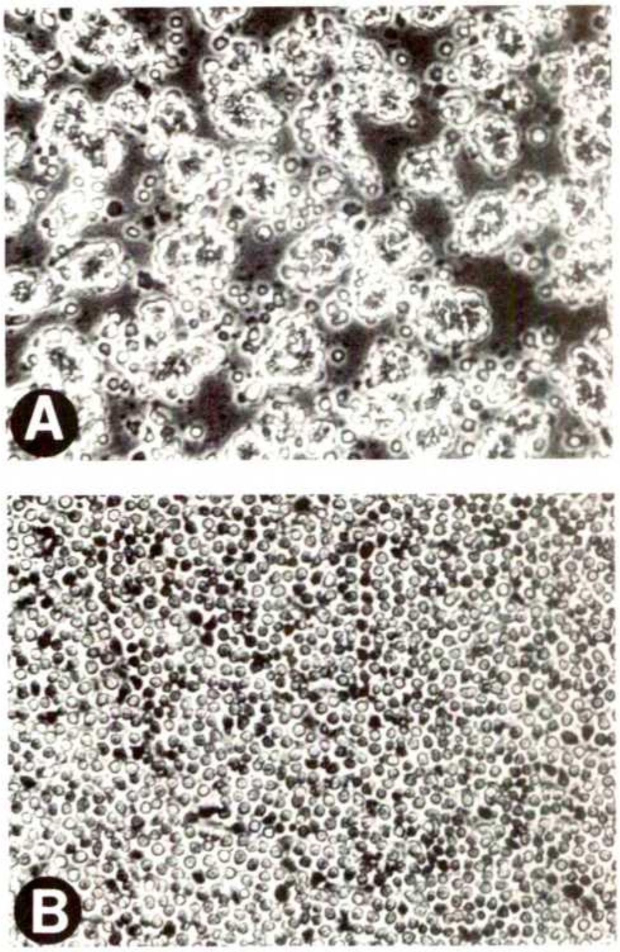

Fig 1. Phase photomicrographs of monolayers of neutrophils after incubation (60 minutes, 37 °C) with (A) 3.3×10^{-7} mol/L of PMN7C3 or (B) control anti-neutrophil IgG 31D8. In the presence of PMN7C3, virtually all the neutrophils have aggregated in clusters. These photomicrographs are from a representative experiment of ten, each using granulocytes from a different donor.

Table 1. Monoclonal Antibody-Induced Leukoagglutination

Antibody	Leukoagglutination*
Anti-PMN7Ag†	
7C3	3-4+
95G8	4+
43B9	2-3+
74D8	4+
66B7	4+
80C9	4+
Control‡	
31D8	0
13F6	0-2+
M1/70	0
41C5	0
8C7	0-1+
55F3	0

*Plates were scored for formation of cell clusters by two observers blinded to the daily protocol (0, homogeneous monolayer; 4+, all cells aggregated in clusters). Data represent the range of values recorded in at least five experiments with each antibody, using granulocytes isolated from different donors. The two groups of antibodies differ significantly in their ability to induce leukoagglutination ($P < .001$, Wilcoxon rank-sum analysis).

†Antibodies that bind to the same antigen as PMN7C3 as detected by Western blotting pattern and competitive binding studies.

‡Monoclonal antibodies binding to other neutrophil antigens.

same antigen as detected by Western blotting pattern and competitive binding studies.[2] These five antibodies also induce PMNs to leukoagglutinate. In contrast, six control monoclonal antibodies binding to other neutrophil antigens did not induce leukoagglutination ($P < .001$, Wilcoxon rank-sum analysis). Control antibodies included M1/70 (anti-Mac 1) which recognizes an antigen associated with the C3bi receptor,[12,13] but does not compete with PMN7C3 for binding in a crossed radioimmunoassay. The ability of different antibodies to induce leukoagglutination did not correlate with the amount of each which binds to the cell surface as detected by ELISA (data not shown). These results suggest that leukoagglutination is not due to passive crosslinking of cells coated with multivalent ligands.

Binding of PMN7C3 to neutrophils induces shape changes and increased migration. Immunoflourescence microscopy has demonstrated that after binding to neutrophils, PMN7C3 undergoes capping to the uropod of the cell.[2] The time course of other cellular events which follow binding of PMN7C3 was examined using time-lapse videotapes recorded through a phase microscope. A sequence of still photographs taken from a representative experiment is presented in Fig 2. Monolayers of adherent PMNs were formed by preincubation of cells in HBSS at 37 °C (Fig 2a). Cellular changes became evident 30 to 40 seconds after the addition of PMN7C3 (3.3×10^{-7} mol/L) to the buffer. Initially cells became less rounded, and many assumed a triangular shape (Fig 2b). This was quickly followed by formation of pseudopods, with a rapid increase in the rate of migration (Fig 2c). Within two to three minutes of the addition of PMN7C3, small clusters of cells started to form (Fig 2d). These changes did not occur after addition of control antineutrophil IgG (PMN31D8) to the incubation medium.

PMN7C3 is both chemoattractant and chemokinetic. The effect of PMN7C3 on neutrophil migration was quantitated by modification of the leading front assay described by Zigmond and Hirsch.[8] When used in the chemoattractant well, PMN7C3 induced a significant increment in migration in concentrations $\geq 3.3 \times 10^{-9}$ mol/L (Fig 3). This effect increased in a dose-dependent fashion with antibody concentrations up to 3.3×10^{-8} mol/L. Higher concentrations of PMN7C3 in the chemoattractant well did not produce any further increment in neutrophil migration. The directed migration induced by 10^{-7} mol/L of PMN7C3 (74.7 ± 3.5 μm) was similar to that produced by an equimolar concentration of fNLLP (69.5 ± 2.5 μm). In contrast, monoclonal IgG binding to other neutrophil antigens (PMN31D8, M1/70) did not induce increased migration (Fig 4). Similar results were obtained when chemotaxis was assayed by migration of PMNs under 1% agarose (data not shown).

To differentiate between chemokinetic (increased random migration) and chemotactic (increased directed migration) effects of PMN7C3, we performed a checkerboard analysis as originally described by Zigmond and Hirsch.[8,9] In these experiments, varied concentrations of PMN7C3 were added to both upper (PMN) and lower (chemoattractant) wells to alter the gradient across the filter. These results are summarized in Table 2. As the concentration of PMN7C3 in the lower wells was increased (vertical columns), neutrophil migration increased in a dose-dependent fashion. Reversal of the gradient by addition of increasing concentrations of PMN7C3 to the upper wells (horizontal rows) inhibited migration of neutrophils into the filter. These data suggest that PMN7C3 is a chemoattractant. Exposure of neutrophils to PMN7C3 in the absence of a chemotactic gradient (diagonal row) also produced a dose-dependent increase in neutrophil migration, although of a smaller magnitude than that seen in the presence of a gradient across the filter. Thus, PMN7C3 also induces chemokinesis.

PMN7C3 inhibits chemotactic response to ZAS and fNLLP. Exposure of neutrophils to a chemotactic stimulus such as *N*-formyl-met-leu-phe (fMLP) may inhibit subsequent response to the formyl peptide and to other chemotactic agents, a process which has been termed deactivation.[14-16] To examine the effect of PMN7C3 on response to other chemoattractants, neutrophils were pretreated with the antibody (6.7×10^{-7} mol/L) for 15 minutes at 4 °C and then washed to remove unbound IgG. Migration of pretreated neutrophils toward ZAS or fNLLP was then measured. Chemotaxis toward both agents over a range of concentrations was significantly inhibited by pretreatment with PMN7C3 (Fig 5). Five other monoclonal antibodies that bind to the same antigen as PMN7C3 had a similar effect on chemotaxis toward ZAS, inhibiting migration to 22.8% ± 3% of control (mean ± SEM, $P < .001$). In contrast, six monoclonal antibodies binding to other neutrophil antigens did not inhibit chemotaxis (mean migration 94.6% ± 17% of control). The degree of inhibition of chemotaxis by PMN7C3 was dependent on the concentration of monoclonal antibody used to pretreat the neutrophils. Inhibition was seen with antibody concentrations as low as 6.7×10^{-9} mol/L (Fig 6).

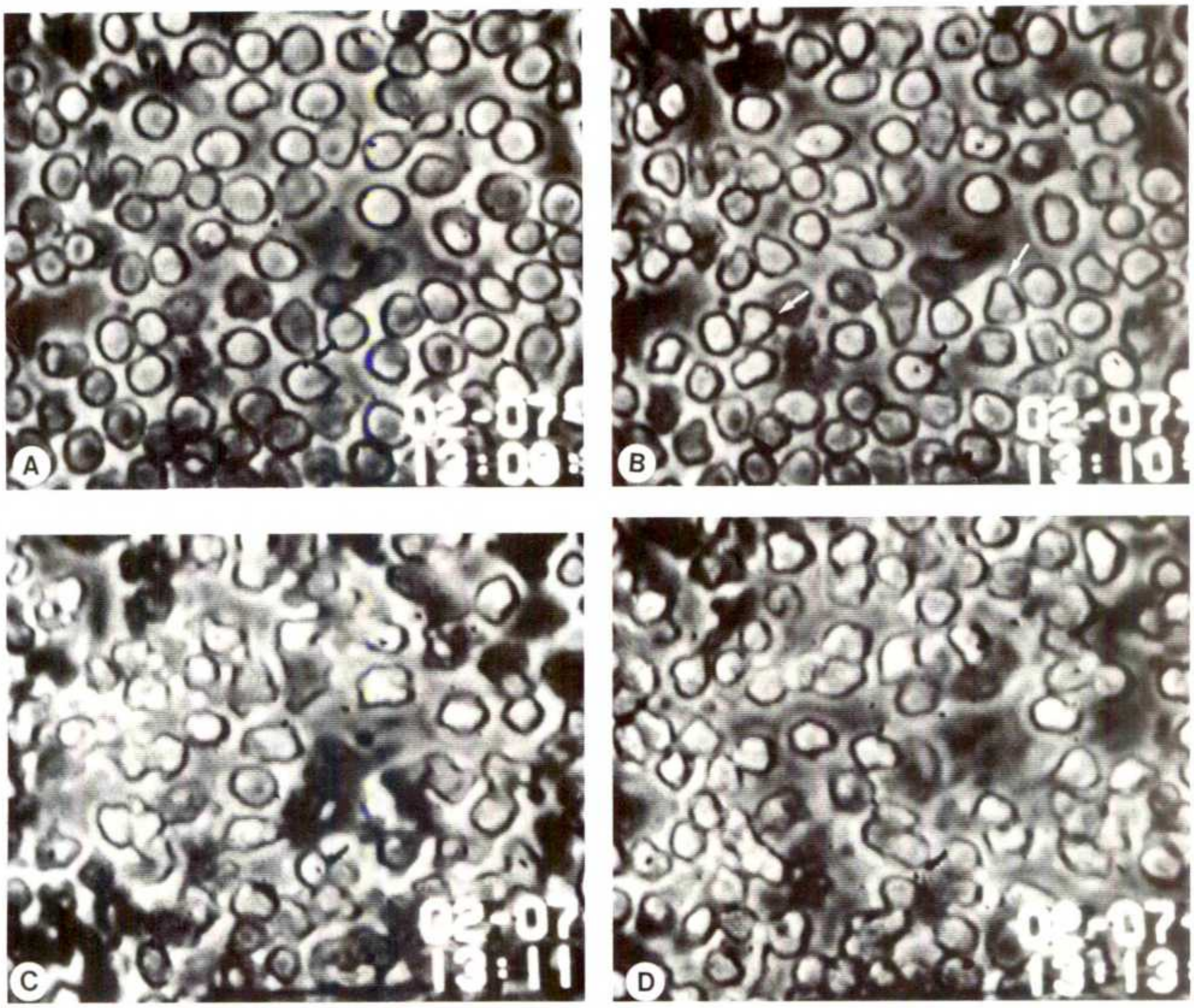

Fig 2. Phase photomicrographs demonstrating the effect of PMN7C3 on a monolayer of adherent neutrophils. Sequential frames were taken from a time-lapse videotape of a single microscopic field (approximate magnification 100×). (A) Polymorphonuclear leukocytes (PMNs) adherent to plastic after incubation (15 minutes, 37 °C) in Hank's balanced salt solution (HBSS) 3.3×10^{-7} mol/L of PMN7C3 was added to the buffer at 13:10:10; (B) 31 seconds after addition of PMN7C3, most neutrophils have become irregular in shape, some assuming a triangular configuration (arrows). (C) Within 95 seconds, most PMNs have formed pseudopods. A marked increase in cellular movement is evident on continuous recordings. (D) After 3.5 minutes, small clusters of cells have started to form. Rapid migration did not cease with the formation of cellular clusters. Neutrophils instead continued to move, frequently leaving one cluster to join another. These data are representative of four independent experiments.

DISCUSSION

In prior work, we demonstrated that binding of PMN7C3 IgG and $F(ab)'_2$ to neutrophils stimulates a transient rise in cytosolic calcium concentration[3] but does not trigger a respiratory burst.[1,2] The present study demonstrates that PMN7C3 activates other cellular responses, including shape change, leukoagglutination, chemotaxis, and chemokinesis. The leukoagglutination reaction appears to be an active process which requires incubation at 37 °C and an intact cytoskeleton. It is not due to passive crosslinking of cells coated with multivalent ligands, since the reaction did not occur after treatment of PMNs with several monoclonal antibodies binding to other surface antigens. Antigen specificity was also demonstrated in the chemotaxis experiments. PMN7C3, but not control monoclonal antibodies, stimulated directed migration at concentrations as low as 3.3×10^{-9} mol/L. At higher concentration (10^{-7} mol/L), the antibody induced a chemotactic response equivalent to that of fNLLP at equimolar concentration. The checkerboard analysis (Table 2) shows that PMN7C3 is chemokinetic as well as chemotactic. Time-lapse videomicroscopy experiments (Fig 2) indicate that changes in cell shape and motility become evident within 30 to 40 seconds after the addition of PMN7C3 to the incubation medium. This coincides with the peak elevation in cytosolic calcium concentration induced by PMN7C3.[3]

These data suggest that changes in intracellular calcium concentration and cell movement and adhesion can be stimu-

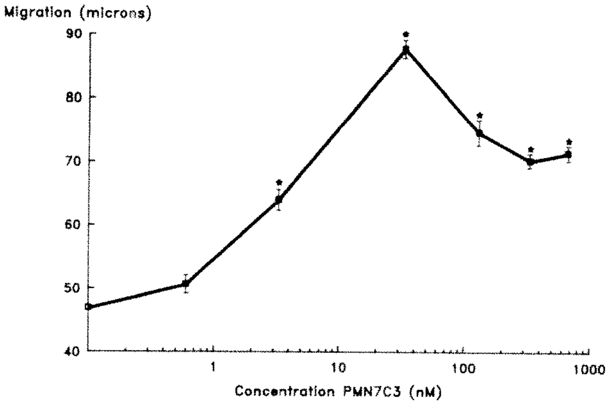

Fig 3. Dose-response curve demonstrating the effect of PMN7C3 as a chemoattractant in a multiwell chemotaxis assembly. Migration was measured as the distance from the top of the filter to the last focal plane containing two polymorphonuclear leukocytes (PMNs). Each point represents the mean ± SEM of data from n = 8 filters from two independent experiments. PMN7C3 induced a significant increment in directed migration at concentration ≥ 3.3 × 10^{-9} mol/L (indicated by stars, $P < .01$, Student's *t* test). In parallel wells, neutrophils migrated 69.5 ± 2.5 μm toward 10^{-7} mol/L *N*-formyl norleucyl leucyl phenylalanine.

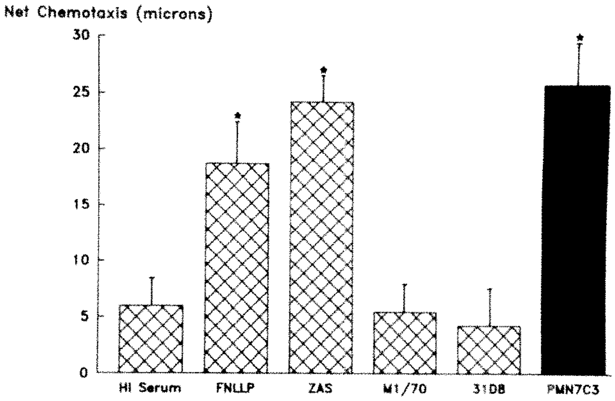

Fig 4. Chemotaxis by neutrophils toward PMN7C3 and control anti-neutrophil IgG (31D8, M1/70). Each value represents the mean ± SEM of data from n = 6 filters from two independent experiments. Random migration has been subtracted. Stars indicate values that differed significantly ($P < .025$, Student's *t* test) from the negative control (heat-inactivated serum). Monoclonal antibodies were used at 6.7 × 10^{-7} mol/L, formylated peptide at 10^{-7} mol/L, and zymosan-activated serum at a 1:5 dilution.

lated without activation of the neutrophil respiratory burst. There are other examples of dissociation of these responses. At low concentrations (10^{-9} to 10^{-10} mol/L), fMLP produces a pattern of cell activation like that seen with PMN7C3.[17,18] Similarly, arachidonate metabolites such as leukotriene B_4 stimulate changes in cellular calcium pools and chemotaxis but are weak activators of the respiratory burst.[15,19-22] Conversely, phorbol myristate acetate (PMA), which binds to protein kinase C,[23] strongly stimulates the respiratory burst without a rise in $(Ca^{2+})_i$.[17,24] Unlike the formylated peptides, PMN7C3 does not stimulate superoxide or hydrogen peroxide production even when used at high concentration. In fact, pretreatment with PMN7C3 inhibits the respiratory burst of neutrophils stimulated with serum-opsonized zymosan.[1,2] Taken together, these facts suggest that activation events leading to cell movement and adhesion can be uncoupled from those leading to the respiratory burst. Such independent pathways would provide a mechanism by which neutrophils could be activated to migrate to sites of inflammation prior to generation of toxic mediators of the inflammatory response. The relationship of PMN7C3-mediated activation to potential transduction events such as leukotriene generation and phosphoinositide turnover is currently under investigation.

Pretreatment with PMN7C3 inhibited subsequent chemotactic response to both ZAS and fNLLP. Exposure to formylated peptides (10^{-7} to 10^{-6} mol/L) has a similar effect on neutrophil response.[14,16] Fehr and Dahinden have suggested that such "deactivation" may be the result of increased adherence of stimulated neutrophils to their substrate with inhibition of subsequent migration.[25] Neutrophil aggregation may have a similar nonspecific effect. Although we cannot rule out such possibilities, they seem unlikely since the checkerboard analysis (Table 2) indicates that PMN7C3 is itself chemokinetic. Furthermore, PMN7C3-treated cells release normal amounts of superoxide in response to formylated peptide,[1] suggesting that functionally competent receptors remain accessible on the cell surface. Thus, PMN7C3 can both stimulate and deactivate chemotactic response without parallel effects on fMLP-induced respiratory burst.

As noted above, PMN7C3 binds to an oligosaccharide antigen which is associated with the C3bi receptor and the related glycoproteins LFA-1 and p150,95 on the neutrophil plasma membrane.[2] An attractive hypothesis is that the functional effects of PMN7C3 are mediated by the C3bi

Table 2. Chemokinetic and Chemotactic Activity of PMN7C3

Concentration of PMN7C3 in Lower Compartment (μg/mL)	Concentration of PMN7C3 in Upper Compartment (μg/mL)				
	0	1	10	50	100
1	48.3 ± 1.2	36.0 ± 3.0*	29.7 ± 1.8	24.0 ± 1.1	23.0 ± 2.6
10	65.5 ± 2.5	56.5 ± 3.5	42.5 ± 2.5	32.5 ± 0.5	25.5 ± 3.5
50	73.7 ± 4.0	80.5 ± 2.5	73.5 ± 1.5	59.0 ± 3.0	47.0 ± 3.0
100		74.0 ± 3.0	64.0 ± 0.0	54.5 ± 2.5	50.0 ± 2.0

*Migration was measured as the distance (microns) from the top of the filter to the last focal plane containing two neutrophils. PMN7C3 was added in the indicated concentrations to the lower (chemoattractant) or (upper polymorphonuclear) leukocytes (PMNs) wells of the chemotaxis assembly. Data represent the mean ± SEM of values from two experiments.

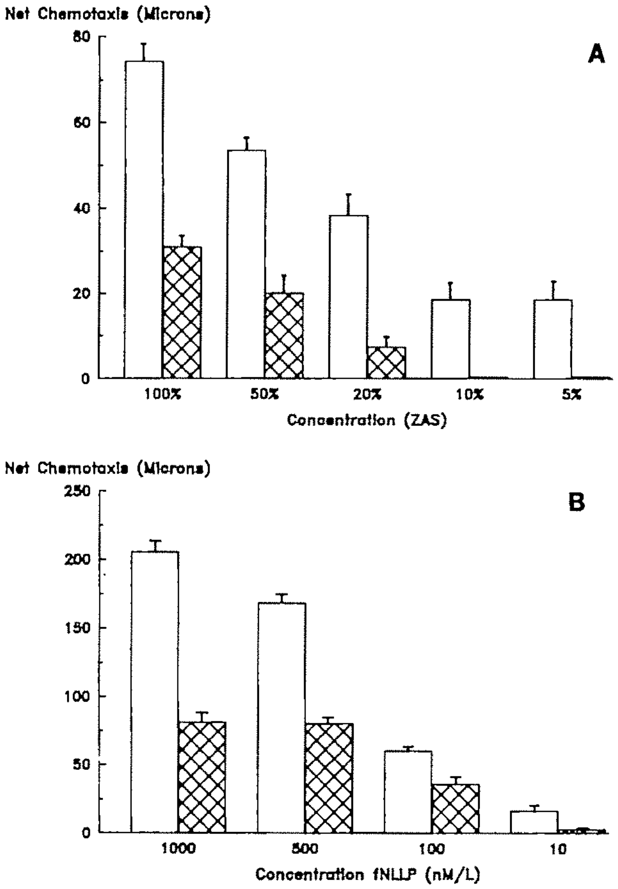

Fig 5. Effect of pretreatment of neutrophils with 6.6 × 10^{-7} mol/L PMN7C3 on chemotaxis toward (A) zymosan-activated serum or (B) *N*-formyl norleucyl leucyl phenylalanine. Polymorphonuclear leukocytes (PMNs) were exposed to monoclonal antibody for 15 minutes at 4 °C and washed to remove unbound IgG prior to assay by migration under agarose. Chemotaxis by PMN7C3-treated neutrophils (hatched bars) was significantly less than that by buffer-treated neutrophils (open bars) at each concentration of chemoattractant tested ($P < .001$, Student's *t* test). Data are the mean ± SEM of n = 18 wells from three independent experiments.

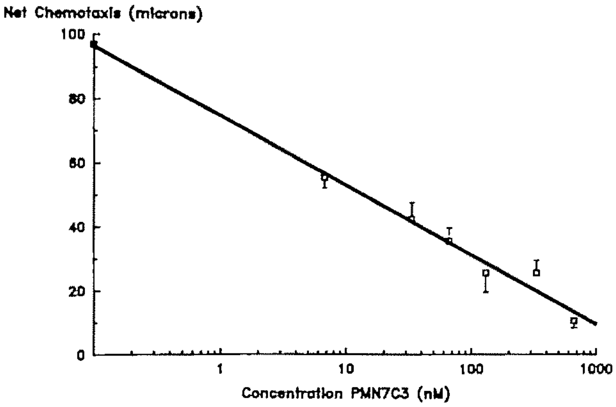

Fig 6. Dose-response curve demonstrating the effect of pretreatment of neutrophils with increasing concentrations of PMN7C3 on chemotaxis toward 10^{-7} mol/L of fNLLP. Chemotaxis was assayed by migration under agarose; each point represents the mean ± SEM of values from 18 wells from three independent experiments. Chemotaxis was significantly inhibited ($P < .001$) at each concentration of PMN7C3 tested.

receptor, which has been postulated to play an important role in PMN-target adhesion.[26-31] Activation of another receptor (insulin) by a polyclonal antiserum has been demonstrated.[32] Our data do not definitively address this question, however. The antigen recognized by PMN7C3 is shared by other cellular proteins and glycolipids.[2] We cannot rule out that the effects of PMN7C3 are due to modulation of one of these other antigen-bearing structures. Another monoclonal antibody recognizing an antigen on CR3 (M1/70) did not mimic the effect of PMN7C3. This does not rule out the possibility that PMN7C3 is affecting cell function through the C3bi receptor, since monoclonal antibodies binding to different sites on receptors can vary in their functional effects.[13,33] Moreover, PMN7C3 stimulates cellular responses (adhesion, motility) which are abnormal in neutrophils lacking C3bi receptors[34-38] and which are inhibited by several monoclonal antibodies binding to the α-subunit of the receptor complex.[13,33,37-39] Recently, another monoclonal antibody (WEM-G1) binding to the same oligosaccharide antigen as PMN7C3 has been demonstrated to stimulate antibody-dependent cytotoxicity of neutrophils toward tumor targets, another funciton that is abnormal in CR3/LFA-1–deficient leukocytes.[37,40,41]

Whether or not PMN7C3 is activating neutrophils through the C3bi receptor and related glycoproteins, our data suggest that this monoclonal antibody may be a useful probe with which to study the cellular events which lead to neutrophil movement and aggregation or to activation of the respiratory burst.

ACKNOWLEDGMENT

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REFERENCES

1. Nauseef WM, Root RK, Newman SL, Malech HL: Inhibition of zymosan activation of human neutrophil oxidative metabolism by a mouse monoclonal antibody. Blood 62:635, 1983

2. Melnick DA, Nauseef WM, Markowitz SD, Gardner JP, Malech HL: Biochemical analysis and subcellular localization of a neutrophil-specific antigen, PMN-7, involved in the respiratory burst. J Immunol 134:3346, 1985

3. Apfeldorf WJ, Melnick DA, Meshulam T, Rasmussen H, Malech HL: A transient rise in intracellular free calcium is not a sufficient stimulus for respiratory burst activation in human polymorphonuclear leukocytes. Biochem Biophys Res Commun 132:674, 1985

4. Boyum A: Isolation of mononuclear cells and granulocytes from human blood: Isolation of mononuclear cells by one centrifugation and of granulocytes by combining centrifugation and sedimentation at 1g. Scand J Clin Lab Invest 21:77, 1967 (suppl 19)

5. Lalezari P, Bernard GE: An isologous antigen-antibody reac-

tion with human neutrophils, related to neonatal neutropenia. J Clin Invest 45:1741, 1966

6. Lalezari P, Radel E: Neutrophil-specific antigens: Immunology and clinical significance. Semin Hematol 11:281, 1974

7. Falk W, Goodwin RH, Leonard EJ: A 48-well chemotaxis assembly for rapid and accurate measurement of leukocyte migration. J Immunol Methods 33:239, 1980

8. Zigmond SH, Hirsch JG: Leukocyte locomotion and chemotaxis. J Exp Med 137:387, 1973

9. Sobel JD, Gallin JI: Polymorphonuclear leukocyte and monocyte chemoattractants produced by human fibroblasts. J Clin Invest 63:609, 1979

10. Nelson RD, Quie PG, Simmons RL: Chemotaxis under agarose: A new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J Immunol 115:1650, 1975

11. Nelson RD, Fiegel VD, Simmons RL: Chemotaxis of human polymorphonuclear neutrophils under agarose: Morphologic changes associated with the chemotactic response. J Immunol 117:1676, 1976

12. Beller DI, Springer TA, Schreiber RD: Anti-Mac-1 selectively inhibits the mouse and human type three complement receptor. J Exp Med 156:1000, 1982

13. Sanchez-Madrid F, Simon P, Thompson S, Springer TA: Mapping of antigenic and functional epitopes on the alpha and beta subunits of two related mouse glycoproteins involved in cell interactions, LFA-1 and Mac-1. J Exp Med 158:586, 1983

14. Ward PA, Becker EL: The deactivation of rabbit neutrophils by chemotactic factor and the nature of the activatable esterase. J Exp Med 127:693, 1968

15. Sha'afi RI, Molski TFP, Borgeat P, Naccache PH: Deactivation of the effects of f-Met-Leu-Phe and leukotriene B_4 on calcium mobilization in rabbit neutrophils. Biochem Biophys Res Commun 103:766, 1981

16. Donabedian H, Gallin JI: Deactivation of human neutrophil chemotaxis by chemoattractants: Effect on receptors for the chemotactic factor f-met-leu-phe. J Immunol 127:839, 1981

17. Korchak HM, Vienne K, Rutherford LE, Wilkenfeld C, Finkelstein MC, Weissmann G: Stimulus response coupling in the human neutrophil II. Temporal analysis of changes in cytosolic calcium and calcium efflux. J Biol Chem 259:4076, 1984

18. Showell JH, Freer RJ, Zigmond SH, Schiffman E, Aswanikumar S, Corcoran B, Becker EL: The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. J Exp Med 143:1154, 1976

19. Ford-Hutchinson AW, Bray MA, Doig MV, Shipley ME, Smith MJH: Leukotriene B, a potent chemokinetic and aggregating substance released from polymorphonuclear leukocytes. Nature 286:264, 1980

20. Naccache PH, Molski TFP, Becker EL, Borgeat P, Vallerand P, Sha'afi RI: Specificity of the effect of lipoxygenase metabolites of arachidonic acid on calcium homeostasis in neutrophils. J Biol Chem 257:8608, 1982

21. Sha'afi RI, Naccache PH, Molski TFP, Borgeat P, Goetzl, EJ: Cellular regulatory role of leukotriene B_4: Its effect on cation homeostasis in rabbit neutrophils. J Cell Physiol 108:401, 1981

22. Shak S, Perez HD, Goldstein IM: A novel dioxygenation product of arachidonic acid possesses potent chemotactic activity for human polymorphonuclear leukocytes. J Biol Chem 258:14948, 1983

23. Niedel JE, Kuhn LJ, Vandenbark GR: Phorbol diester receptor copurifies with protein kinase C. Proc Natl Acad Sci USA 80:36, 1983

24. Sha'afi RI, White JR, Molski TFP, Shefcyk J, Volpi M, Naccache PH, Feinstein MB: Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. Biochem Biophys Res Commun 114:638, 1983

25. Fehr J, Dahinden C: Modulating influence of chemotactic factor-induced cell adhesiveness on granulocyte function. J Clin Invest 64:8, 1979

26. Lay WH, Nussenzweig V: Receptors for complement on leukocytes. J Exp Med 128:991, 1968

27. Ehlenberger AG, Nussenzweig V: The role of membrane receptors for C3b and C3d in phagocytosis. J Exp Med 145:357, 1977

28. Mantovani B: Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. J Immunol 115:15, 1975

29. Newman SL, Johnston RB Jr: Role of binding through C3b and IgG in polymorphonuclear neutrophil function: Studies with trypsin-generated C3b. J Immunol 123:1839, 1979

30. Ross GD, Lambris JD: Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils, and erythrocytes. J Exp Med 155:96, 1982

31. Sanchez-Madrid F, Nagy JA, Robbins E, Simon P, Springer TA: A human leukocyte differentiation antigen with distinct alpha subunits and a common beta subunit: The lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1), and the p150/95 molecule. J Exp Med 158:1785, 1983

32. Simpson IA and Hedo JA: Insulin receptor phosphorylation may not be a prerequisite for acute insulin action. Science 223:1301, 1984

33. Wright SD, Rao PE, Van Voorhis WC, Craigmyle LS, Iida K, Talle MA, Westberg EF, Goldstein G, Silverstein SC: Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. Proc Natl Acad Sci USA 80:5699, 1983

34. Arnaout MA, Pitt J, Cohen HJ, Melamed J, Rosen FS, Colten HR: Deficiency of a granulocyte-membrane glycoprotein (gp150) in a boy with recurrent bacterial infections. N Engl J Med 306:693, 1982

35. Dana N, Todd RF, Pitt J, Springer TA, Arnaout MA: Deficiency of a surface membrane glycoprotein (Mo1) in man. J Clin Invest 73:153, 1984

36. Arnaout MA, Spits H, Terhorst C, Pitt J, Todd RF: Deficiency of a leukocyte surface glycoprotein (LFA-1) in two patients with Mo1 deficiency: Effects of cell activation on Mo1/LFA-1 surface expression in normal and deficient leukocytes. J Clin Invest 74:1291, 1984

37. Kohl S, Springer TA, Schmalstieg FC, Loo LS, Anderson DC: Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. J Immunol 133:2972, 1984

38. Springer TA, Thompson WS, Miller LJ, Schmalsteig FC, Anderson DC: Inherited deficiency of the MAC-1, LFA-1, p150,95 glycoprotein family and its molecular basis. J Exp Med 160:1901, 1984

39. Arnaout MA, Todd RF, Dana N, Melamed J, Schlossman SF, Colten HR: Inhibition of phagocytosis of complement C3- or immunoglobulin G-coated particles and of C3bi binding by monoclonal antibodies to a monocyte-granulocyte membrane glycoprotein. J Clin Invest 72:171, 1983

40. Lopez AF, Vadas MA: Stimulation of human granulocyte function by monoclonal antibody WEM-G1. Proc Natl Acad Sci USA 81:1818, 1984

41. Spitalnik PF, Spitalnik SL, Lopez AF, Vadas MA: Monoclonal antibody WEM-G1 stimulates granulocyte function and recognizes a carbohydrate antigen. Clin Res 33:354A, 1985 (abstr)

Induction of Growth Alterations in Factor-Dependent Hematopoietic Progenitor Cell Lines by Cocultivation With Irradiated Bone Marrow Stromal Cell Lines

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Mary Ann Sakakeeny, Katherine Wagner, Joe Falco, T.J. FitzGerald, and Joel S. Greenberger

We studied the production of hemopoietins by x-irradiated plateau-phase cultures of cloned marrow stromal cell lines derived from C3H/HeJ marrow, termed D2XRII and clone 11. The production of CSF in agar overlay of control or 10,000 rad irradiated stromal cultures was quantitated by induction of colonies in: overlaid fresh marrow, IL-3–dependent cell line 32D cl 3, or GM-CSF/IL-3–dependent cell lines FDCP-1 or bg/bg cl 1. Conditioned media were tested for CSF by bioassay using fresh marrow cells, for M-CSF (CSF-1) by RIA, and for IL-3 and GM-CSF by microwell proliferation assay with 32D cl 3 and FDCP-1 cells, respectively. X-irradiation to doses that decreased CSF-1 to 40% of control levels (>5,000 rad) resulted in a 30-fold increase in growth of FDCP-1 or bg/bg cl 1 cells in liquid co-culture or agar culture overlay with no detectable growth of 32D cl 3. The frequency of subculture of nonautocrine, factor independent (FI) variant clonal lines of FDCP-1 or bg/bg cl 1 cells was increased over 1000-fold by 15 weeks cocultivation with irradiated stromal cell cultures. FI subclonal lines formed tumors in syngeneic mice and contained no detectable poly A messenger RNA for GM-CSF or IL-3, and no elevation in c-*myc*, c-*abl*, c-*src*, or *erb*-B onc gene-specific messenger RNA compared to parent factor-dependent lines. These data indicate that x-irradiated plateau phase marrow stromal cells produce increased levels of cell contact-mediated biologically active hemopoietin(s) other than M-CSF, GM-CSF, or IL-3 and induce nonautocrine factor-independent malignant cell lines in vitro.

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THE mechanism of leukemogenesis in patients exposed to alkylating agents and x-irradiation is unknown. It is unclear whether leukemogenesis results from direct alteration of the hematopoietic stem cell, indirect alteration of the stromal supporting cells of the marrow, or by a combination of mechanisms.

Regulation of hematopoiesis is mediated in part by a group of specific glycoproteins termed colony-stimulating factors (CSFs).[1] At least four distinct murine CSFs have been categorized according to the colonies that are stimulated in agar culture: M-CSF (macrophage colony-stimulating factor, CSF-1),[2] GM-CSF (granulocyte-macrophage colony-stimulating factor),[1] G-CSF (granulocyte colony-stimulating factor),[1] and multi-CSF (IL-3).[1,3,4] In addition to induction of mixed granulocyte-erythroid-macrophage-megakaryocyte colonies in vitro, termed CFU-GEMM,[1,3,4] IL-3 has been shown to be unique for maintenance of specific hematopoietic cell lines.[5–9] Each CSF has been purified to homogeneity and GM-CSF and multi-CSF have been molecularly cloned and expressed in vitro.[9,10]

Another mechanism controlling hematopoiesis, including the totipotential stem cell population, is referred to as the hematopoietic inductive microenvironment, and is mediated via supporting stromal cells of the bone marrow.[11–13] The adherent cell layer in long-term bone marrow culture (LTBMC) has been shown to function as an in vitro counterpart of the in vivo microenvironment.[11–13] Hematopoiesis in vitro in LTBMC is dependent on the presence of an adherent stromal cell layer.[11–13] Some recent data suggest that these stromal cells are the source of other non-CSF growth factors that regulate hematopoiesis in vitro and in vivo.[12,13]

Recently, it has been reported that exposure of unseparated LTBMCs to x-irradiation in vitro results in a significant increase in detectable GM-CSF released by the adherent layer.[12,13] The mechanism of this phenomenon has been attributed to removal of inhibitor cells,[12] decreased consumption of CSF by hematopoietic cells,[13] or a combination of mechanisms. X-ray induction of existing or new growth factors has remained an additional but untested possibility.

In the present studies, permanent marrow-derived cloned stromal[14] and hematopoietic cell lines[6,7] were used to dissect the mechanism of the alterations in growth factor production by x-irradiation. The results show that x-irradiation of clonal stromal cell lines significantly enhances the growth of factor-dependent hematopoietic cell lines in vitro. This growth enhancement is greatly facilitated by cell contact.

MATERIALS AND METHODS

Mice. C3H/HeJ and C57BL/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Me.

Stromal cell lines. The methods for establishment of an adherent stromal cell line D2XRII and C3H/HeJd156 cl 11 have been previously published.[14] These cell lines were isolated from nonadherent cell populations derived from C3H/HeJ long-term bone marrow cultures and were subcloned in Dulbecco's Modified Eagle's Medium (Gibco, Long Island, NY).

Both adherent cell lines, when plated at 2×10^6 cells into 25 cm^2 tissue culture flasks (Falcon, Oxnard, Calif), grew to a dense confluent monlayer within 3 to 4 days and were maintained in Dulbecco's medium at 33 °C in a high-humidity incubator with 7% CO_2. The cells were passaged weekly by trypsinization (trypsin 0.25%, Gibco) and replating.

Hematopoietic cell lines. The establishment of permanent factor-dependent hematopoietic progenitor cell lines FDCP-1 and 32D

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cl 3 have been previously described in detail.[6,7] Another factor-dependent cell line was derived from C57BL/6 bg/bg marrow cultures designated bg/bg cl 1. Lines 32D cl 3 and bg/bg cl 1 were grown in suspension culture in McCoy's 5A modified medium (Gibco) containing 15% fetal calf serum (Hazelton, Vienna, Va) and 10% WEHI-3 CM as a source of IL-3.[3] FDCP-1 cells were maintained in RPMI medium (Gibco) containing 15% fetal calf serum (Hazelton) and 5% WEHI-3 CM.[6] All cell lines were maintained in 33 °C in a high-humidity incubator with 7% CO_2 and were fed biweekly.

X-irradiation of stromal cell lines. Confluent plateau-phase cultures of D2XRII and C3H/HeJ cl 11 cells were x-irradiated by a 250 kVp orthovoltage unit as previously described, at a constant dose rate of 200 rad per minute.[14] Cultures were agar overlaid (as described below) immediately after irradiation, or were trypsinized and transferred into 35 × 10 mm tissue culture dishes (Falcon) at high density (5×10^6 cells per dish) to re-establish an adherent monolayer, and agar was overlaid on the following day. Another set of controls and 10,000 rad x-irradiated cultures was maintained at 33 °C and cell-free conditioned medium was harvested weekly and stored at −20 °C.

Detection of colony-stimulating factors (CSF) in the conditioned medium. Fresh bone marrow, CBA mouse fetal liver cells,[1] or FDCP-1, 32D cl 3, and bg/bg cl 1 cell lines were washed three times in serum-free medium and resuspended in fresh medium with increasing concentrations of the CM harvested from control or irradiated D2XRII or cl 11 stromal cell cultures, containing 0.3% agar in 1 mL in 35 × 10 mm tissue culture dishes (Falcon). Colonies of ≥ 50 cells were scored on days 7, 14, and 21. Fresh marrow cells were obtained from C3H/HeJ mice on the day of the experiment. Single cells were similarly resuspended in the conditioned media containing 0.3% agar in 1 mL volume in 35 × 10 mm tissue culture dishes and colonies scored on day 7. After scoring, the entire agar layer was removed, air dried on a glass slide, and stained with Wright's Giemsa, mixed esterase stain, or acetylcholine esterase stain.[14]

For further morphologic studies of the types of cells stimulated by each conditioned medium, CBA fetal liver cells were prepared in Dulbecco's modified Eagle's medium containing 20% heat-inactivated human plasma, 0.3% agar, and 0.1 mL of the conditioned medium in 1 mL volume in 35 × 10 mm tissue culture dishes. Cultures were incubated at 37 °C in a high-humidity incubator with 10% CO_2 and colonies of ≥ 50 cells were scored on day 7. To detect the possible ability of the conditioned media to initiate, but not sustain, colony formation by other types of progenitor cells, replicate cultures were removed from the incubator after two days and 0.1 mL of pokeweed mitogen spleen-conditioned medium (PM-SCM) was added as a source of IL-3.[3,4] After a further incubation period of five days, colonies of ≥ 50 cells were scored. After scoring, all the cultures were fixed in situ by adding 1.0 mL of 2.5% with Luxol-Fast-Blue and counterstained with hematoxylin.

For the proliferation assay, 5×10^4 cells of each line, 32D cl 3, and FDCP-1 were washed and resuspended in serial tenfold dilutions of the conditioned medium harvested from control and x-irradiated D2XRII and clone 11 cultures in microwells (0.1 mL volume). After 24 hours of incubation, the cells were pulsed with ^3H-thymidine (Amersham) and harvested 4 hours later.

Cell-free conditioned medium from both stromal cell lines D2XRII and clone 11 was also tested for CSF-1 by radioimmunoassay (RIA) as previously described.[15]

Northern blot hybridization analysis of GM-CSF– and IL-3–specific messenger RNA expression and of c-abl, c-myc, erb-b, and c-src oncogene transcription. Total cellular RNA was isolated by a modification of the guanidine hydrochloride extraction method as described by Adams et al.[16] Poly-A messenger RNA was prepared according to published methods.[25] RNAs containing IL-3 and GM-CSF sequences were identified by hybridization with 2.0×10^6 cpm/mL nick-translated probes (5×10^8 cpm/ug). The probes used were a 1.1 kb Eco R1 fragment of a mouse IL-3 clone and a 1.1 kb Eco R1 fragment of GM-CSF,[10] courtesy of Dr T. Burgess, Ludwig Institute for Cancer Research, Victoria, Australia. Hybridizations were performed at 42 °C for 24 hours in 50% formamide and 5 × SCC, followed by washing at 46 °C in 0.1 × SCC and 0.1% SDS as described previously.[17,18]

For oncogene transcript detection, 10 ug of RNA per lane was applied to formaldehyde 1% agarose gel and transferred to nitrocellulose filters as described.[17,18] Hybridization was performed with ^{32}p-labeled nick-translated probes at 42 °C in buffer containing 50% formamide and 3 × SSC. Filters were washed at 48 °C in 0.1 × SSC. The probes used were mouse c-*myc* cDNA fragment and mouse c-*abl* cDNA fragment (1.8 kb, HicII-XhoI), each generously provided by Y. Ben-Nerish, Whitehead Institute, M.I.T., Cambridge, Mass; v-*src* from Rous Sarcoma virus (RS-A) cloned in pUC-13, generously provided by B. Mathey-Prevot Whitehead Institute, M.I.T., Cambridge, Mass; *erb*-B cross-reactive mouse EGF receptor (2.4 kb EcoRi fragment from genomic clone of NIH-3T3 cells) generously provided by Mien-Chie Hung, Whitehead Institute, M.I.T., Cambridge, Mass.

G-band karyotype preparation. Karyotyping was carried out according to published methods.[7]

Tumorgenicity studies. Aliquots of 1×10^7 or 1×10^6 cells of each cloned cell line were injected subcutaneously into the right flank of adult syngeneic C57BL/6J (for bg/bg cl 1) or B6D2F1 (for FD-CP-1) mice. Mice were examined weekly for tumors at the site of injection. Dead or dying mice were subjected to complete autopsy according to published methods.[27]

RESULTS

Colony formation in agar by factor-dependent hematopoietic progenitor cell lines. FDCP-1, 32 D cl 3, and C57BL/6J bg/bg cl 1 cells were tested for colony formation in agar in WEHI-3 CM. The cells were plated at increasing density from $1 \times 10^3 - 1 \times 10^5$ cells/mL in response to serial dilution of WEHI-3 CM. Lines bg/bg cl 1, 32D cl 3, and FDCP-1 formed dense colonies, detected as early as four days after plating; however, 32D cl 3 cells had a higher plating efficiency in 0.1% WEHI-3 CM. Thus, 1×10^4 and 1×10^3 cells per 1.0 mL dish and 10% WEHI-3 CM were used as positive controls for comparable counting of colonies (Fig 1).

Irradiated cloned stromal cell lines stimulate colony formation by GM-CSF/IL-3–dependent hematopoietic progenitor cell lines in agar overlay. Confluent plateau-phase stromal cell cultures were overlaid with either fresh marrow cells, 32D cl 3, or bg/bg cl 1 target cells at increasing plating densities, with no WEHI-3 CM added. As shown in Table 1, fresh marrow cells formed 278 ± 25 colonies per 10^5 cells in WEHI-3 CM, and 253 ± 36 and 192 ± 14 colonies per 10^5 cells when overlaid on confluent D2XRII or clone 11 stromal cell cultures, respectively. 32D cl 3 cells formed 2307 ± 242 colonies per 10^4 cells plated in agar in the presence of WEHI-3 CM as a source of IL-3. No 32D cl 3 colonies were detected at 10^4 or 10^5 per 1.0 mL over control or irradiated monolayers of D2XRII or clone 11 cells during 21 days of incubation. FDCP-1 and bg/bg cl 1 cells formed 2 ± 0.5 and 7 ± 0.6 colonies per 10^4 cells, respectively, over control

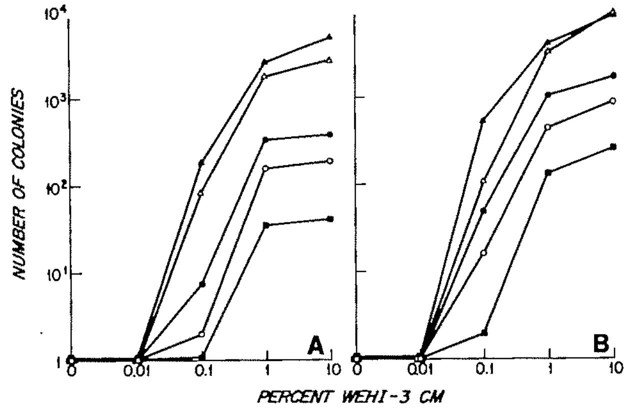

Fig 1. Colony formation at day 7 in 0.3% agar of (A) FDCP-1 and (B) 32D cl 3 cells in response to tenfold dilutions of WEHI-3CM. Cells were plated at: (■), 1 × 10^3; (O), 5 × 10^3; (●), 1 × 10^4; (△), 5 × 10^4; and (▲), 1 × 10^5 cells per 35 × 10mm plate in 1.0 mL. Results are presented as the mean of 12 dishes in three experiments. The standard error of the mean was <10%.

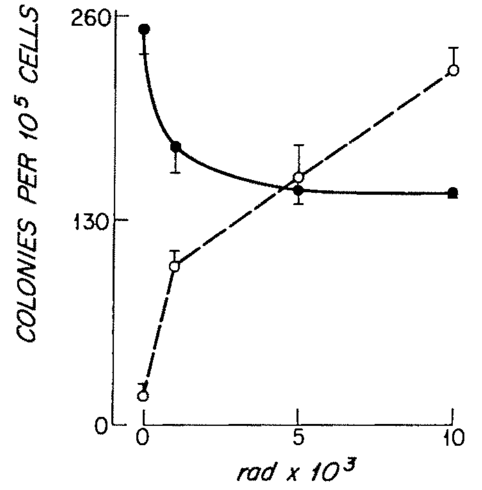

Fig 2. Induction of fresh bone marrow colonies (●) and FDCP-1 cell line colonies (O) by D2XRII stromal cells following x-irradiation. 2 × 10^5 fresh marrow or FDCP-1 cells per 1.0 mL were overlaid in 0.3% agar on irradiated stromal cultures and colonies of ≥50 cells were scored after seven days. Results are presented as mean ± SEM for eight cultures in two experiments.

D2XRII monolayers, and each cell line formed 1 colony per 10^4 cells over clone 11. Thus, there was a low but clearly detectable growth of FDCP-1 and bg/bg cl 1 but not 32D cl 3 cells in agar overlay of D2XRII or clone 11 stromal cells.

As shown in Table 1, 10,000 rad x-irradiation of the D2XRII stromal cell line resulted in a decrease in the number of colonies scored on day 7 by fresh marrow target cells, from 253 ± 36 (control) to 149 ± 11 colonies per 1 × 10^5 cells plated. A less significant decrease was observed with clone 11 irradiated stromal cells overlaid with fresh marrow (Table 1). In contrast, the number of FDCP-1 and bg/bg cl 1 cell colonies increased following irradiation of D2XRII cells to 17.1 ± 2 and 16.5 ± per 1 × 10^4 cells plated, respectively. FDCP-1 cells formed 15 ± 2 colonies and bg/bg cl 1 cells formed 63.3 ± 3 colonies per 10^4 cells when overlaid in agar over irradiated clone 11 stromal cells (Table 1). The results were similar if control stromal cell lines D2XRII and clone 11 were transferred to 30 × 10 mm dishes and then irradiated. The separation of effects of x-irradiation of D2XRII cells comparing agar overlay results with FBM (decrease in colonies) and FD cells (increase in colonies) was most prominent at x-ray doses over 5,000 rad (Fig 2).

Quantitation of CSF in the conditioned medium from x-irradiated D2XRII cells. Conditioned medium from control or irradiated stromal cell cultures was harvested weekly over 5 weeks. Table 2 shows the results of the bioassays performed by adding 10% final concentrations of the conditioned medium harvested at week 1 following irradiation of the stromal cultures to single-layer agar cul-

Table 1. Bioassay for Agar-Overlay Detection of Colony-Stimulating Factor (CSF) Released by Cloned Stromal Cell Lines D2XRII and Clone 11

	Number of Colonies† in Agar Overlay With Cell Targets (plating density/mL)			
Source of CSF*	FBM (1 × 10^5)	32D cl 3 (1 × 10^4)‡	FDCP-1 (1 × 10^4)	bg/bg cl 1 (1 × 10^4)
D2XRII-control	253 ± 36	<1	2 ± 0.5	7 ± 0.5
D2XRII-10,000 rad irradiated	149 ± 11	<1	17 ± 1.2	16 ± 5.5
Clone 11-control	192 ± 14	<1	1 ± .05	1.3 ± 0.5
Clone 11-10,000 rad irradiated	187 ± 14	<1	15 ± 2	63.3 ± 3
WEHI-3 CM (10%)	278 ± 25	2302 ± 242	757 ± 176	223.2 ± 31
L-Cell CM (10%)	128 ± 11	<1	<1	<1
No underlayer	<1	<1	<1	<1
No CM	<1	<1	<1	<1

*Control and x-irradiated plateau-phase stromal cultures were trypsinized and replated at 5 × 10^6 cells to 35 × 10 mm tissue culture dishes and overlaid on the following day.

†Colonies of ≥50 cells were scored after seven days of incubation at 37 °C. Each number represents the mean ± SEM of eight dishes in two separate experiments.

‡The number of cells plated was 1 × 10^3 per dish and data are adjusted to 1 × 10^4/dish for comparison.

Table 2. Bioassay for Detection of Colony-Stimulating Factor (CSF) in Stromal Cell–Conditioned Medium*

Conditioned Medium	Number of Colonies in Agar† (plating density/mL)				
	FBM (1×10^5)	FDCP-1 (1×10^4)	32D cl 3 (1×10^4)	bg/bg cl 1 (1×10^4)	Fetal Liver (2×10^4)
WEHI-3 CM	208 ± 18	757 ± 176	2302 ± 242	223.2 ± 31	NT
D2XRII Control	198 ± 17	<1	<1	<1	37 ± 9
D2XRII 10,000 rad x-irradiated	176 ± 12	<1	<1	<1	42 ± 7
Clone 11 control	272 ± 10	<1	<1	<1	77 ± 21
Clone 11 10,000 rad x-irradiated	225 ± 37	<1	<1	<1	42 ± 2
MLCM‡ (GM-CSF)	NT‖	NT	NT	NT	15 ± 4
SCM§ (Multi-CSF) + GM-CSF)	NT	NT	NT	NT	61 ± 2

*Conditioned medium harvested from control and 10,000 rad x-irradiated stromal cultures was added to a mixture of medium and agar with various target cells at final 10% dilution.

†Colonies were scored after seven days. Each number represents the mean ± SE of 6 plates in three separate experiments.

‡Mouse lung conditioned medium.

§Spleen conditioned medium.

‖Not tested.

tures containing fresh marrow cells, FDCP-1, 32D cl 3, bg/bg cl 1, or fetal liver cells. Conditioned medium failed to induce detectable colony formation over 21 days by FDCP-1, 32D cl 3, or bg/bg cl 1 cells. There was also no detectable IL-3 in the CM by microwell assay using 32D cl 3 target cells. Control unirradiated stromal-cell CM induced 198 ± 17 (for D2XRII) and 272 ± 10 (for clone 11) colonies by 10^5 fresh marrow target cells. Individual colonies were picked and stained and were found to be 80% to 85% composed of macrophages and 10% to 15% mixed granulocyte-macrophages.

The level of CSF-1 measured by RIA four weeks after x-irradiation decreased to 40% of the control level (796.3 U/mL compared to the control level of 1968.6 U/mL). This decrease was less significant than that measured by the nonspecific bioassay which detects GM-CSF, G-CSF, and multi-CSF, as well as CSF-1 (78.0 units at week 4 after 10,000 rad compared to a control level of 97.4 U/0.1 mL). Similar results were obtained in bioassay and CSF-1 radioimmunoassay with CM harvested from control and irradiated clone 11.

Fetal liver cells formed colonies in conditioned medium from control D2XRII cultures to a level of 37 ± 9 per 2×10^4 cells plated, and to a level of 77 ± 21 cells plated when tested with CM from clone 11 cultures. The morphology of the fetal liver colonies showed macrophage, GM, and pure granulocyte colonies (Table 3). Thus, FD lines did not detectably respond to irradiated stromal cell CM, while fresh marrow and fetal liver cells continued to respond and form colonies in agar.

The colony formation by fetal liver cells grown in CM from irradiated or control stromal cell lines might have been attributed to CSF-1 that is present at relatively high levels in the medium, or to GM-CSF (Table 2). The colonies induced

Table 3. Morphology of Fetal Liver Cell–Derived Colonies Induced by Conditioned Medium From Irradiated Cloned Stromal Cell Lines*

Conditioned Media	IL-3 Added Day 2	Percentage of Colonies					
		G	GM	M	Eos	E	Mixed-E
D2XRII control	−	6	8	86	0	0	0
	+	10	16	69	5	0	0
D2XRII 10,000 rad irradiated	−	3	6	91	0	0	0
	+	9	5	72	14	0	0
Clone 11 control	−	10	7	83	0	0	0
	+	12	26	55	4	0	3
Clone 11 10,000 rad irradiated	−	7	12	81	0	0	0
	+	11	13	70	2	2	2
MLCM† (10%)	−	16	10	40	24	0	0
	+	16	21	38	7	6	12
SCM‡ (10%)	−	12	18	34	5	11	20

*Fetal liver cells were plated in a plasma/agar mixture as described in Materials and Methods in the presence of a 10% final concentration of stromal cell CM.

Colonies were scored and then fixed and strained for differential scoring of cell types (M, macrophage; E, erythroid).

†Mouse lung cell–conditioned medium.

‡Spleen cell–conditioned medium.

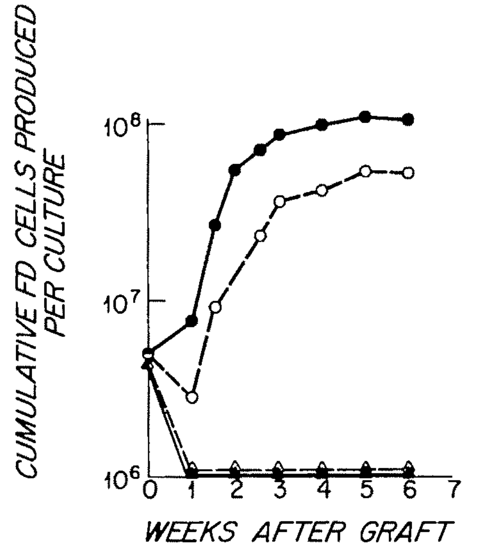

Fig 3. Cumulative FDCP-1 or 32D cl 3 cells harvested weekly from control or 10,000 rad x-irradiated D2XRII cocultures. Confluent D2XRII cultures were overlaid in liquid culture with 5 × 10^6 cells in 8.0 mL of FDCP-1 or 32Dcl 3 cells. All nonadherent cells were removed once weekly by medium change. (△) 32D cl 3 + control D2XRII; (▲) 32Dcl3 + 10,000 rad irradiated D2XRII; (○) FDCP-1 + control D2XRII; or (●) FDCP-1 + 10,000 rad irradiated D2XRII.

by stromal cell CM were primarily multicentric macrophage colonies typical of those stimulated by CSF-1 (Table 3). Spleen-conditioned medium (SCM) containing IL-3 was next added to replicate fetal liver cell cultures after two initial days of incubation with stromal cell CM. After a further incubation period of five days, all the cultures were fixed and stained as described. While no erythroid, eosinophil, or multipotential hematopoietic progenitor cell colonies were induced by the stromal cell line CM alone, addition of IL-3 on the second day resulted in formation of detectable eosinophil, erythroid, and mixed-erythroid colonies. The effect was most pronounced with 10,000 rad irradiated clone 11–conditioned medium. The data suggested the presence of either low levels of GM-CSF that could have allowed survival of cells that could respond to IL-3 two days later, or to a growth factor other than GM-CSF or CSF-1 in the stromal cell line CM.

Table 5. Derivation of Factor-Independent Clonal Cell Lines From FDCP-1 Cells Following Cocultivation With Irradiated D2XRII Bone Marrow Stromal Cell Line

Stromal Cell Culture	No. FI Variant Clones Obtained/No. Tested* Weeks in Culture					
	3	5	6	7	8	15
D2XRII control	0/50	0/30	0/10	0/30	0/30	0/500†
D2XRII 10,000 rad irradiated	0/50	5/15‡	8/8	NT	5/5	18/20

*Cultures of plateau-phase D2XRII cells were overlaid the day after 10,000 rad irradiation with 1 × 10^6 FDCP-1 cells. All nonadherent cells were then removed weekly. NA cells were plated in 0.3% agar at 1 × 10^5 or 1 × 10^4 cells/mL in the absence of WEHI-3 CM. All harvests formed over 100 colonies/10^3 cells plated in WEHI-3 CM. Results are for those cultures generating > 1/10^5 colonies in the absence of added WEHI-3 CM and which generated a permanent FI line following explant and subculture of nonadherent cells to single-cell micro wells. Denominator represents total number of single-cell microwells tested at that weekly harvest.

†At week 15, microwell assays of 1 cell per well were performed with NA cells harvested from control and irradiated D2XRII coculture. There were no wells generating a cell line out of 500 single-cell wells plated from the control coculture cell harvest, while 18 of 20 single-cell wells from irradiated cocultures generated a cell line in the absence of WEHI-3 CM. NT – not tested.

‡One of these clones is FDCP-1 cl 39, shown in Table 4.

Mixed suspension cultures of FDCP-1 or bg/bg cl 1 with irradiated stromal cells induce factor-independent variant lines. D2XRII cultures were next established in 25 cm^2 tissue culture flasks. Plateau-phase cultures were then either exposed to a single dose of 10,000 rad or left unirradiated. The medium was aspirated and 5 × 10^6 FDCP-1, bg/bg cl 1, or 32D cl 3 cells were added in 8 mL of RPMI medium supplemented with 15% FCS with no added WEHI-3 CM or other source of IL-3 or GM-CSF. Microscopic examination of the mixed cultures revealed attachment of FDCP-1 and bg/bg cl 1 cells to the stromal layer and formation of multiple "cobblestone" areas.[11] No such attachment was seen with 32D cl 3 cells.

FDCP-1 and bg/bg cl 1 cells grew at a rapid rate and the effect was more accelerated over irradiated D2XRII cultures (Fig 3). In contrast, no viable 32D cl 3 cells were detected after 2 weeks in the mixed cultures (Fig 3). There was a rapid increase in the cumulative number of the cells, and both groups reached plateau at week 5 (Fig 3). Similar

Table 4. Effect of Coculture With Stromal Cells on Colony Formation by FDCP-1 Cells in Varying Concentrations of WEHI-3 CM

Cells*	Number of Colonies per 1 × 10^4 Cells (day 7) WEHI-3 CM Added				
	0	0.1%	.1%	1%	10%
FDCP-1	<1	<1	14 ± 4	661.5 ± 44	1081.5 ± 311
FDCP-1 cocultured 4 weeks with D2XRII control cells	<1	9.5 ± 1	152.5 ± 41	448 ± 29	615 ± 27
FDCP-1 cocultured 4 wks with 10,000 rad irradiation D2XRII cells	<1	8.2 ± 4	123.2 ± 5	682 ± 133	476.6 ± 68
FDCP-1 clone 39† FI line	625 ± 146	1327 ± 265	>2000	>2000	>2000

*Cells were plated in 1.0 mL Petri dishes at 1 × 10^3 or 1 × 10^4 per dish in each final concentration of WEHI-3 CM and containing 0.3% agar. Colonies of ≥50 cells were scored at day 7. Results are extrapolated to number per 10^4 when scored at 10^3/dish for very high colony numbers. Results are the mean ± SEM of at least 8 plates in 3 separate experiments.

†FDCP-1 cl 39 was derived from a single cell removed at week 5 after coculture with irradiated D2XRII stromal cells.

results were obtained using clone 11 instead of D2XRII cells or using bg/bg cl 1 cells instead of FDCP-1 cells.

Beginning at week 3, FDCP-1 cells in mixed cultures were tested for growth dependence on WEHI-3–conditioned medium. Table 4 shows results of a representative assay performed on week 4 of one experiment. Colonies in agar formed by FDCP-1 cells removed after 4 weeks co-culture with either irradiated or control D2XRII cultures and were detected at or above a 0.1% concentration of WEHI-3 CM.

Factor-independent growth of FDCP-1 cell colonies in agar was not detected after up to 15 weeks cocultivation with control D2XRII cultures (Table 5). In contrast, FDCP-1 cells harvested after 5 weeks or longer coculture with irradiated D2XRII cells grew in the absence of WEHI-3 CM (Table 5). Similar results were obtained from mixed cultures containing D2XRII and bg/bg cl 1 cells, and mixed cultures containing clone 11 with FDCP-1 or bg/bg cl 1 cells.

Northern blot hybridization analysis of IL-3 and GM-CSF mRNA levels in factor-independent clones. In an attempt to elucidate the mechanism by which FDCP-1 and bg/bg cl 1 clones became factor-independent, conditioned media harvested from dense cultures of representative clonal factor-independent cell lines was first assayed for detectable levels of IL-3 and GM-CSF in microwell assay, using as target cells clonal lines of factor-dependent FDCP-1, 32D cl 3, and bg/bg cl 1 cells. There was no detectable growth of any of these IL-3–dependent or dual GM-CSF/Il-3–dependent lines.

We next tested extracted poly A messenger RNA from aliquots of 10^8 cells of each factor-independent variant clonal line for detectable levels of IL-3 or GM-CSF–specific mRNA by northern blot hybridization with IL-3 or GM-CSF–specific cDNA probes. As shown in Fig 4, none of the independent cell lines tested showed detectable levels of IL-3

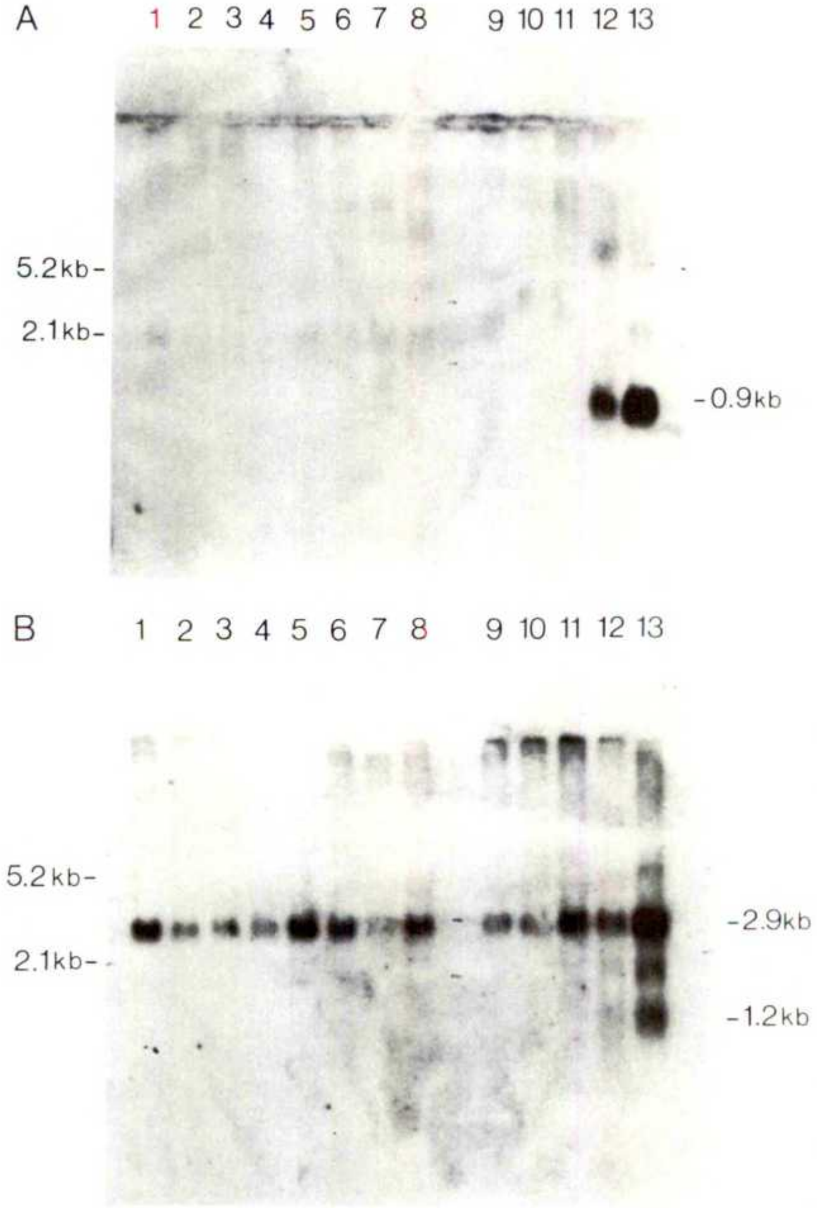

Fig 4. Northern blot hybridization of cellular poly-A selected messenger RNA from cloned cell lines with nick-translated probes of (A) murine IL-3 or (B) GM-CSF as described in Materials and Methods. Results are for cellular RNA from: FDCP-1 parent line (lane 1); FDCP-1 factor-independent clone 39 (lane 2); bg/bg cl 1 parent line (lane 3); bg/bg factor-independent clone 49 (lane 4). Lanes 5–11 are each of 7 additional factor-independent cell lines; for panel A WEHI-3 clone 3 cellular RNA (lane 12) and WEHI-3 clone 3 poly-A selected RNA (lane 13). The same cellular poly-A mRNAs are used in panel B. The positive control for GM-CSF is RNA from TPA-stimulated cell line EL4 generously provided by Dr Dov Pluznik, Rehovot Israel (lane 12) and poly-A selected RNA from EL4 (lane 13). The long length GM-CSF probe used includes an exon from an adjacent gene coding for an mRNA of 2.9 Kb that is present in many cell types.[10] The GM-CSF–specific position of this probe failed to detect any GM-CSF mRNA (1.2 Kb) in the transformed cell lines.

or GM-CSF mRNA. In contrast, mRNA from the GM-CSF–producing line EL4 and the IL-3–producing line WEHI-3 showed high levels of both GM-CSF and IL-3 mRNA, respectively (Fig 4).

Expression of oncogene mRNA in factor-independent clonal cell lines. We next assayed seven representative FI variant clones and their parent lines (FDCP-1 and bg/bg cl 1) for expression of c-*myc*, c-*abl*, c-*src*, and *erb*-B mRNA. As shown in Fig 5, each of the cell lines showed expression of c-*myc* mRNA. None of the cell lines expressed detectable c-*src* or *erb*-B mRNA. Although there was some variation in the level of c-*myc* RNA transcription, high expression was found in the parent FDCP-1 and bg/bg cl 1 cell lines as well as in their factor-independent clones (Fig 5). Furthermore, c-*abl* RNA transcription was present in all the cell lines tested. The c-*abl* RNA transcripts in the bg/bg cell line and its independent clones were significantly higher than those in RNA from the FDCP-1 line and its independent clones.

Test for malignancy of factor-independent variant lines. Karyotypes prepared from metaphases of five factor-independent variant clones of bg/bg cl 1 and the parent factor-dependent line were analyzed. The parent bg/bg cl 1 line had 36 to 38 chromosomes and was XO. Each of the factor-independent clones had a new and distinct karyotype. New or marker chromosomes did not appear in the parent factor-dependent line tested over 6 months' passage in WEHI-3 CM.

Each factor-independent clone of bg/bg cl 1 or FDCP-1 and each factor-dependent parent line was tested for tumorgenicity in syngeneic mice. None of twenty 33 gram B6D2F1 mice injected with 1×10^7 parent line FDCP-1 cells developed tumors at the injection site after 6 months. In contrast, 11 of 20 mice injected with 1×10^6 factor-independent FDCP-1 (subclone FI #39) developed >2 cm tumors at the injection site. None of 15 adult C57BL/6J mice injected with 1×10^7 cells of parent line bg/bg cl 1 developed tumors at the site of injection after 8 months. In contrast, >2.5 cm diameter tumors were detected at the site of injection within 8 months in 7 of 10 mice injected with 1×10^6 cells of line bg/bg independent clone 5 and 9 of 10 mice injected with 1×10^6 cells of line bg/bg independent clone 9.

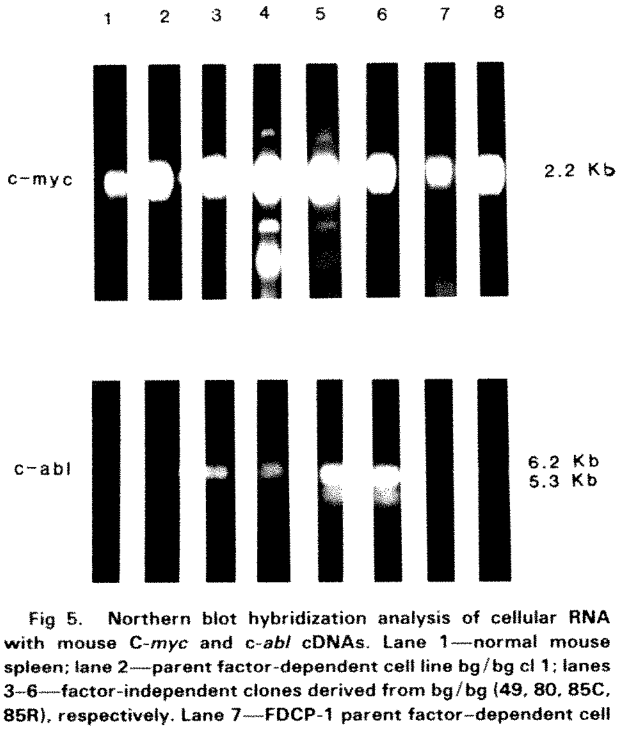

Fig 5. Northern blot hybridization analysis of cellular RNA with mouse C-*myc* and c-*abl* cDNAs. Lane 1—normal mouse spleen; lane 2—parent factor-dependent cell line bg/bg cl 1; lanes 3–6—factor-independent clones derived from bg/bg (49, 80, 85C, 85R), respectively. Lane 7—FDCP-1 parent factor-dependent cell line; lane 8—FDCP-1 factor-independent clone (39).

DISCUSSION

Murine marrow-derived stromal cell lines constitutively produce CSF-1, as measured by induction of macrophage colonies by overlaid fresh marrow target cells, and by radioimmunoassay for CSF-1 in the cell free-supernatant.[14,15] Following x-irradiation, these stromal cell lines produce another novel hematopoietic stimulatory factor(s) distinct from IL-3 and GM-CSF by biologic assays. Preliminary experiments with poly-A mRNAs purified from control or 10,000 rad irradiated D2XRII cells also failed to detect IL-3– or GM-CSF–specific mRNA by molecular hybridization.

The radiation-induced factor stimulates proliferation of dual GM-CSF/IL-3–dependent hematopoietic progenitor cell lines FDCP-1 and bg/bg cl 1 but not IL-3–dependent line 32D cl 3 very effectively in liquid cocultivation, less effectively in agar overlay with 0.3% agar, and markedly less in 0.5% agar overlay. Furthermore, cocultivation induces evolution of factor-independent malignant variant cell lines. In other experiments freshly isolated factor-dependent bg/bg–derived cell lines with normal karyotype also responded to irradiated stromal cell overlay by colony formation in agar and generated factor-independent cell lines. That the phenomena described here are not unique to the D2XRII and clone 11 stromal cell lines is suggested by our preliminary experiments using irradiated primary purified fresh murine stromal cell cultures[14] that also induced evolution of malignant factor-independent variants of bg/bg cl 1 cells. Thus, the release of this factor(s) is increased by x-irradiation under conditions where CSF-1 production is decreased and its biologic effect is greatly enhanced by cell contact.

That the present FI lines were not induced by an "autocrine" mechanism[20] was shown by: (1) Lack of IL-3 or GM-CSF in CM derived from the independent cell lines when tested in a proliferation assay with 32D cl 3 or FDCP-1 target cells, and (2) no detectable intracellular IL-3 or GM-CSF Poly A mRNA in the FI clones by Northern blot hybridization with specific cDNA probes.

Transformation of factor-dependent to factor-independent cells has been associated with malignant change. Such phenotypic changes have been attributed to transforming growth factors[20,22] (TGFs) released by various tumor cell lines or to the action of retroviral gene products.[21,22,24-26] Membrane-bound PDGF-2 coded by v-*sis* has recently been described.[31] Transfer of mutagenic stimuli from one cell to other nontreated cells has been described with UV irradiation of human fibroblastic cells in vitro.[23] Plateau-phase bovine aortic endothelial cells exposed to supralethal Co^{60}-gamma rays show increased superoxide dysmutase levels.[27]

Plasminogen activator production has been shown to be increased by fibroblasts following exposure to supralethal doses of ultraviolet light (UV) or the tumor promoter TPA.[28,29] UV light or nitrosylurea exposure of human fibroblasts have been found to induce factors capable of stimulating protein synthesis.[30] Our data may prove to be explained by x-ray induction of a transforming growth factor.

Malignant transformation of factor-dependent cells has also been associated with activation of proto-oncogenes, either in single [24,25] or sequential activation.[26] Malignant transformation of the FDCP-1 cell line by the Abelson virus associated c-*abl* gene was recently described.[24] Since the transcripts of c-*myc* and c-*abl* in both our parent bg/bg cl 1 and FDCP-1 cell lines and their factor-independent clones were quantitatively and qualitatively similar, we cannot attribute the observed growth factor independence to altered expression of either of these genes. However, we cannot rule out the possibility that mutations or other alterations may have occurred in one or more of the genes giving rise to altered or activated proteins.

The present system of coculture of factor-dependent hematopoietic cell lines with irradiated stromal cells should prove to be a valuable system for analysis of the cellular interactions involved in the normal and leukemogenic hematopoietic microenvironment.

ACKNOWLEDGMENT

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REFERENCES

1. Metcalf D: The granulocyte-macrophage colony stimulating factors. Science 229:16, 1985

2. Stanley ER, Heard PM: Factors regulating macrophage production and growth: Purification and some properties of the colony-stimulating factor from medium conditioned by mouse L-cells. J Biol Chem 252:4305, 1977

3. Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F, Prystowski MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B: Biological properties of homogenous Interleukin-3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P-cell stimulating factor activity, colony-stimulating factor activity and histamine-producing cell stimulating factor activity. J Immunol 131:282, 1983

4. Greenberger JS, Humphries RK, Reid DM, Messner H, Sakakeeny MA: Molecularly cloned and expressed murine T-cell gene product with multipotential CSF properties is biologically similar to Interleukin-3. Exp Hematol 13:249, 1985

5. Greenberger JS: Self-renewal of factor-dependent hematopoietic progenitor cell lines derived from long-term bone marrow cultures demonstrates significant mouse strain genotype variation. J Supramolec Str 13:501, 1980

6. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D: Growth of factor dependent hematopoietic precursor lines. J Exp Med 152:1036, 1980

7. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ, Eckner RJ: Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc Nat Acad Sci USA 80:2931, 1983

8. Metcalf D: Multi-CSF dependent colony formation by cells of a murine hematopoietic cell line: Specificity and action of multi-CSF. Blood 65:357, 1985

9. Fung MC, Hapel AJ, Ymer S, Cohen DR, Johnson RM, Campbell HD, Young IG: Molecular cloning of cDNA for murine Interleukin-3. Nature 307:233, 1984

10. Gough NW, Gough J, Metcalf D, Kelow A, Groel P, Nicola NM, Burgess AW, Dunn AR: Molecular cloning of the DNA encoding a mouse hematopoietic growth regulation, granulocyte-macrophage colony stimulating factor. Nature 309:763, 1984

11. Greenberger JS: Long-term hematopoietic cultures, in Golde D (ed): Methods in Hematology (vol 11). New York, Churchill Livingston, 1984, p 204

12. Gualtieri RJ, Shadduck RK, Baker DG, Quesenberry PJ: Hematopoietic regulatory factors produced in long-term murine bone marrow cultures and the effect of in vitro irradiation. Blood 64:516, 1984

13. Naparstek E, Donnelly J, Kase K, Greenberger JS: Biologic effects of x-irradiation of murine long-term bone marrow cultures on the production of granulocyte-macrophage colony-stimulating factors. Exp Hematol 13:701, 1985

14. Greenberger JS, Klassen V, Kase K, Sakakeeny MA: Effects of low dose rate irradiation on plateau phase bone marrow stromal cells in vitro: Demonstration of a new form of non-lethal physiologic alteration of support of hematopoietic stem cells. Int J Rad Onc Biol Phys 10:1027, 1984

15. Shadduck RK, Waheed A: Development of a radioimmunoassay for colony stimulating factor. Blood Cells 5:421, 1979

16. Adams SL, Sobel ME, Howard BH, Olden K, Yamada KM, DeCrombrugghe B, Pastan I: Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus-transformed chick embryo fibroblasts. Proc Natl Acad Sci USA 74:3399, 1977

17. Hieter PA, Nax EE, Seidman JG, Maizel JV Jr, Leder P: Clones human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 22:197, 1980

18. Thomas DS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201, 1980

19. Hapel AJ, Warren HS, Hume DA: Different colony stimulating factors are detected by the Interleukin-3 dependent cell lines FDC-P1 and 32D cl 23. Blood 64:786, 1984

20. Shrader JW, Crapper RM: Autogenous production as a mechanism for transformation of bone marrow derived cells. Proc Natl Acad Sci USA 80:6892, 1983

21. Twardzik DR, Todaro GJ, Marquardt CO: Transformation induced by Abelson murine leukemia virus involves production of a polypeptide growth factor. Science 216:894, 1982

22. Adkins B, Leutz A, Graf T: Autocrine growth induced by src-related oncogenes in transformed chicken myeloid cells. Cell 39:439, 1984

23. Schorpp M, Mallick U, Rahmsdorf HJ, Herrlich P: UV-induced extracellular factor from human fibroblasts communicates the UV response to nonirradiated cells. Cell 37:861, 1984

24. Cook WD, Metcalf D, Nicola NA, Burgess AW, Walker F: Malignant transformation of a growth factor-dependent myeloid cell line by Abelson virus without evidence of autocrine mechanism. Cell 41:677, 1985

25. Pierce JH, DiFiore PP, Aaronson SA, Potter M, Pumphrey J, Scott A, Ihle JN: Neoplastic transformation of mast cells by Abelson-MuLV: Abrogation of IL-3 dependence by a nonautocrine mechanism. Cell 41:685, 1985

26. Oliff A, Agranovsky O, McKinney MD, Murtey VVS,

Bauchwitz R: Friend murine leukemia virus-immortalized myeloid cells are converted into tumorigenic cell lines by Abelson leukemia virus. Proc Natl Acad Sci USA 82:3306, 1985

27. Rubin DB, Drab EA, Ward WF, Smith LJ, Fowell SM: Enzymatic responses to radiation in cultured vascular endothelial and smooth muscle cells. Rad Res 99:420, 1984

28. Wigler M, Weinstein LB: Tumor promoter induces plasminogen activator. Nature 259:232, 1983

29. Miskin R, Ben-Ishai R: Induction of plasminogen activator by UV light in normal and xeroderma pigmentosum fibroblasts. Proc Natl Acad Sci USA 78:6236, 1981

30. Cohn SM, Krawisz BR, Dresler SL, Lieberman MW: Induction of replicative DNA synthesis in quiescent human fibroblasts by DNA damaging agents. Proc Natl Acad Sci USA 81:4829, 1984

31. Robbins KC, Leal F, Pierce JH, Aaronson SA: The v-sis/PDGF-2 transforming gene product localizes to cell membrane, but is not a secretory protein. The EMBO J 4:1783, 1985

High Fetal Hemoglobin Production in Sickle Cell Anemia in the Eastern Province of Saudi Arabia Is Genetically Determined

By Barbara A. Miller, Mohammed Salameh, Mohammed Ahmed, Jim Wainscoat, Giovanna Antognetti, Stuart Orkin, David Weatherall, and David G. Nathan

Homozygous sickle cell disease in the eastern province of Saudi Arabia is clinically mild. Circulating fetal hemoglobin levels of 16.0 ± 7.4% were found in these anemic patients, but only 1.09 ± 0.97% in their sickle trait parents. To determine whether these sickle cell anemia patients inherit an increased capacity to synthesize fetal hemoglobin, a radioimmunoassay of fetal and adult hemoglobin was performed on erythroid progenitor (BFU-E)-derived erythroblasts from Saudi Arabian sickle cell patients and their parents. Mean fetal hemoglobin content per BFU-E-derived erythroblast from Saudi Arabian sickle cell patients was 6.2 ± 2.4 pg/cell or 30.4 ± 8.6% fetal hemoglobin (normal 1.1 ± 0.7 pg/cell and 5.1 ± 1.8%). Linear regression analysis of % HbF in peripheral blood versus % HbF per BFU-E-derived cell showed a positive correlation with an *r* of 0.65. The variance of the intrinsic capacity to produce HbF may account for almost 40% (r^2) of the variance of circulating fetal hemoglobin but other factors, particularly selective survival of F cells, must also contribute significantly. Despite virtually normal HbF levels in sickle trait parents of these Saudi patients, mean fetal hemoglobin production per BFU-E-derived erythroblast in these individuals was elevated to 3.42 ± 1.79 pg/cell or 16.1 ± 6.4% fetal hemoglobin, and the magnitude of fetal hemoglobin production found in parents correlated with that of the patients. These data indicate that the high fetal hemoglobin in Saudi sickle cell disease is genetically determined but expressed only during accelerated erythropoiesis. Further evidence of such genetic determination was provided by analysis of DNA polymorphisms within the β-globin gene cluster on chromosome 11. This revealed a distinctive 5′ globin haplotype (+ + − + +) on at least one chromosome 11 in all high F SS and AS tested. The precise relationship of this haplotype to HbF production in this population remains to be defined.

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HOMOZYGOUS sickle cell anemia (SCA) in the eastern province of Saudi Arabia is, in general, clinically milder than sickle cell anemia of African origin.[1] High fetal hemoglobin levels averaging 18% to 25% of the total hemoglobin have been found in the former population,[2,3] compared to mean levels of 5% to 10% found in Jamaican or American SCA patients.[4,5] It is thought that the very high fetal hemoglobin levels may contribute to the mildness of the disease.

Both the inherent capacity to produce fetal hemoglobin and the selective survival of F cells[6] contribute to circulating fetal hemoglobin levels in sickle cell anemia.[7] How these influences interact to produce the unusually elevated fetal hemoglobin levels observed in the Saudi population is not known. Conclusive evidence in support of an inherited trait has not been developed. Classical deletion or nondeletion hereditary persistence of fetal hemoglobin is not involved since parents do not have elevated circulating hemoglobin F levels.[2]

To investigate the basis of increased fetal hemoglobin in these patients, the intrinsic capacity of Saudi sickle cell anemia patients and their parents for hemoglobin F synthesis (designated the HbF program) was measured using a radioimmunoassay to assess the amount of fetal hemoglobin produced in vitro by erythroid progenitor-derived erythroblasts.[8,9] This technique measures production uncomplicated by the influence of selection of F cells in vivo. The results indicate that the HbF program is a major contributor to circulating hemoglobin F in these Saudi sickle cell anemia patients. Additional findings show that the high HbF program in Saudi SCA patients is inherited from at least one parent but its expression requires accelerated erythropoiesis. High fetal hemoglobin production in Saudi SCA is also associated with inheritance of a specific pattern of restriction endonuclease sites within the β-globin region of DNA on chromosome 11 spanned by the ϵ and $\psi\beta 1$ globin genes. This pattern of DNA polymorphisms that constitute a 5′ "haplotype" for this region has been associated with increased HbF production in other populations as well.[10-14]

MATERIALS AND METHODS

Blood samples. Blood samples were obtained from Saudi Arabians who are employees of the Arabian American Oil Company (Aramco) or from their dependents. While patients were chosen at random, no one was included who had received a blood transfusion in the past six months, who had a sickle cell crisis, or active infection in the seven days before assay. All patients were older than three years of age. In each family, at least one member had sickle cell anemia. Blood and bone marrow samples were also obtained from American sickle cell patients seen in the Hematology Clinic at Children's Hospital, Boston, Mass, and from normal volunteer donors who gave their informed consent. The samples from Saudi Arabia were drawn in 10% ACD and shipped at 4 °C. All assays were performed within 72 hours.

Analysis of blood. CBC and reticulocyte counts, F cell enumeration by acid elution, hemoglobin electrophoresis on citrate agar and cellulose acetate, and alkali denaturation to determine percent fetal hemoglobin were performed on all samples. Ratio of Gγ to Aγ fetal hemoglobin was determined by Triton X-100 electrophoresis.[15]

Methylcellulose culture of peripheral blood and bone marrow mononuclear cells. Blood and marrow samples were diluted with tissue culture medium (Iscove's Modified Dulbecco's medium = IMDM), layered over Ficoll-hypaque (Pharmacia, Piscataway, NJ), and centrifuged for 20 minutes at 1,200 g at room temperature. The cells at the interface were washed three times. Cells were plated at 1

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to 2×10^5 cells/mL in 35mm petri dishes (Falcon, Oxnard, Calif) in a mixture containing 0.9% methylcellulose, 30% fetal calf serum (Gibco, Grand Island, NY), 9.0 mg/mL deionized BSA (fraction V, Sigma, St. Louis), 1.4×10^{-4} mol/L beta-mercaptoethanol, 5% Mo cell line–conditioned medium as a source of BPA (generously provided by Dr David Golde), and with 2.0 U/mL crude erythropoietin (Toyobo, New York) in IMDM. The plates were incubated in humidified 4% CO_2 at 37 °C for 14 days and colonies were then counted.

Sample preparation for hemoglobin assay. Erythroid colonies were "plucked" with a drawn out Pasteur pipet, pooled in 0.3 to 0.5 mL IMDM, 4 °C, and the number of cells counted. A precisely measured volume was then transferred to a second tube and spun. The pellet was resuspended in a precisely measured volume of 0.5 mg/mL carrier sheep Hb containing 0.02% Na-azide. This suspension was sonicated, microcentrifuged to remove cell debris, and stored at 4 °C. Samples were routinely assayed within 10 days of preparation.

Radioligand immunoassay of HbA and HbF. The radioligand immunoassay of total hemoglobin or hemoglobin F in pg per BFU-E–derived cell or of percent HbF in such cells was performed as previously described.[8] Briefly, adult and fetal hemoglobin standards were prepared from adult blood, cord blood, or sheep blood samples by CCl_4 lysis of washed cells. The adult and cord blood samples were used to construct standard curves and were diluted with 0.5 mg/mL sheep Hb. Sonicated cell extracts derived from erythroid colonies diluted with 0.5 mg/mL sheep Hb were assayed at two to three dilutions. Antihemoglobin F and total antibodies were prepared from guinea pigs injected with cord blood or alkali denatured cord blood.

Linear standard curves were consistently obtained, and the Hb in 3,000 to 10,000 cells was routinely assayed. Reproducibility of this assay has been discussed previously,[9] but in general, duplicate dishes or multiple assays performed on samples from one individual on different days yielded results within 10% of the mean. The calculation of hemoglobin content per BFU-E–derived cell was based as before on the assumption that all such cells are potential F cells.[9]

Significance of differences among the means of n groups (Tables 2 and 3, n = 5 and 3, respectively) was determined by one-way analysis of variance. A priori comparisons of means of any two groups were then performed, using F tests as tests of significance. A probability of less than 0.05 was taken to be statistically significant. Significance of correlation coefficients (Fig 1, n = 17; Fig 3, n = 15) was assessed using a *t* distribution.

Restriction endonuclease analysis of DNA and determination of the regional haplotype. Procedures for DNA isolation, blot hybridization, and probes were as previously described.[16] Only five restriction endonuclease site polymorphisms were studied, all in the 5′ group of the β-globin gene cluster. These included a potential Hinc II site 5′ to the ϵ gene, a Hind III site in the intervening sequence 2 (IVS-2) of the Gγ-globin gene, a Hind III site in the IVS-2 of the Aγ-globin gene, and two Hinc II sites, one in the $\psi\beta 1$ gene and another 3′ to it. The "haplotype" for this region was then established, based on the presence or absence of digestion at these sites.

RESULTS

Characterization of patients. Peripheral blood samples were obtained from Saudi Arabian sickle cell anemia patients and their families who met the criteria discussed in Materials and Methods. The results of blood and reticulocyte counts, hemoglobin electrophoresis, F cell determination, and analysis of percent fetal hemoglobin by alkali denaturation are shown in Table 1 and confirm results published elsewhere.[1,3,17,18] Mean MCVs were low in Saudi Arabian patients, probably secondary to the high incidence of alpha thalassemia trait[17,19,20] and iron deficiency in this region. Fetal hemoglobin levels in the Saudi patients had a mean of 16.0 ± 7.4% compared to 7.1 ± 4.0% for American sickle cell patients of African origin seen in the clinic at Children's Hospital, Boston. Fetal hemoglobin levels in the Saudi AS parents were generally normal but were elevated in a few parents, as demonstrated by the high standard deviation.

Table 1. Hematologic Characteristics of Saudi Arabian Sickle Cell Patients

	AA	AS	SS
Hemoglobin g/dL	11.8 ± 1.4	11.1 ± 2.6	9.33 ± 2.13
% Retic	1.5 ± 0.4	2.19 ± 1.40	6.53 ± 3.8
MCV	69.7 ± 5.4	73.9 ± 10.6	69.9 ± 8.1
F cell number	ND	3.71 ± 6.24	39.6 ± 16.7
% Hemoglobin F	0.87 ± 0.33	1.09 ± 0.97	16.0 ± 7.4
No.	3	51	36

Hematologic characteristics of Saudi Arabian sickle cell patients and families from hemogram, acid elution test, and alkali denaturation. Mean levels ± 1 standard deviation are shown.

Analysis of fetal hemoglobin from eight Saudi Arabian SCA patients by Triton X-100 gel revealed that all patients had more Gγ than Aγ fetal hemoglobin. The mean value was 76.5 ± 5.1% G gamma globin, a finding previously noted in this population.[1] In contrast, 80% of American black patients with sickle cell disease have 40% Gγ and 60% Aγ; only 20% have more Gγ than A.[21]

Hemoglobin F program in BFU-E–derived erythroblasts. Bone marrow samples were not obtained from Saudi patients. Therefore it was necessary to ascertain that the HbF content of peripheral blood BFU-E–derived erythroblasts was representative of the HbF program. To do so, the total hemoglobin and HbF contents of BFU-E–derived erythroblasts were measured in BFU-E–derived colonies cultured from the bone marrow and peripheral blood of normal consenting American donors and American black sickle cell patients. The results shown in Table 2 confirmed the previous findings of Friedman et al.[9] BFU-E–derived erythroid cells of normal donors cultured from marrow or blood did not differ significantly with respect to pg HbF or percent HbF/BFU-E–derived cells. Similarly, no difference was observed between bone marrow and peripheral blood of American sickle cell patients. Furthermore, repeated studies of the peripheral blood BFU-E HbF program carried out in three Saudi patients and one normal volunteer showed that the standard deviation of the assay performed over a two-week period on a single individual was less than 15%. Therefore, it was concluded that the erythroblasts derived from peripheral blood BFU-E could provide a reliable determination of the HbF program in Saudi patients.

Of note was the fact that the pg HbF and % HbF/BFU-E–derived cell were statistically different for BFU-E from the peripheral blood of normal donors, American sickle cell patients, and Saudi sickle cell patients (Table 2).

Influence of the HbF program on peripheral blood fetal hemoglobin in sickle cell disease. The influence of the HbF program on the circulating fetal hemoglobin in these Saudi sickle cell anemia patients is shown in Fig 1. Linear

Table 2. Assessment of HbF in BFU-E–Derived Erythroblasts

Patients	Normal Donor		American SS		Saudi SS
Source	Marrow	Blood	Marrow	Blood	Blood
No. cells × 10^3/BFU-E-derived colony	10.4 ± 6.2	12.3 ± 5.7	2.9 ± 1.5	7.1 ± 3.9	7.2 ± 4.3
pg HbF/BFU-E-derived cell	1.2 ± 0.52	1.3 ± 0.7	3.2 ± 0.9	3.6 ± 1.8*	6.2 ± 2.4†
pg total Hb/BFU-E-derived cell	18.2 ± 2.9	19.6 ± 6.9	14.9 ± 3.0	16.2 ± 6.0	20.8 ± 6.7
% HbF in BFU-E-derived cells	6.8 ± 3.6	6.1 ± 2.1	21.1 ± 3.9	23.4 ± 9.5‡	30.4 ± 8.6§
No.	10	8	3	13	17

BFU-E-derived colony size and radioligand assay of fetal and total hemoglobin per BFU-E-derived cell from cultures of bone marrow and peripheral blood of normal donors and American sickle cell disease patients and from peripheral blood of Saudi Arabian sickle cell disease patients.

*$P < 0.01$ when compared to normal donor, blood.

†$P < 0.001$ when compared to American SS, blood.

‡$P < 0.001$ when compared to normal donor, blood.

§$P < 0.025$ when compared to American SS, blood.

regression analysis of the percentage of fetal hemoglobin in BFU-E–derived cells versus the percent of fetal hemoglobin in peripheral blood provided a significant positive correlation with an r of 0.65. Therefore, the variance of the intrinsic HbF program appears to account for nearly 40% (r^2) of the variance of the circulating fetal hemoglobin in these patients. Other factors, particularly selective survival of F cells, must contribute to the remaining 60%. To determine whether alpha thalassemia affected hemoglobin production in vitro, Saudi sickle cell patients were divided into two groups based on MCV greater than or less than 78. The HbF programs for both groups were not significantly different (data not shown).

Figure 2 shows the relationship of the HbF program in BFU-E–derived cells to the percent fetal hemoglobin in the peripheral blood of the AS parents of the Saudi sickle cell anemia patients. The data demonstrate that no correlation exists between the HbF program and the circulating HbF in these individuals. Most of these β^s heterozygotes were parents of patients with circulating high HbF and, as demonstrated in Fig 3 (discussed below), their HbF programs did correlate with those of their offspring. This relationship was not detected from studies of peripheral blood because nearly all of them had either normal or only slightly elevated circulating HbF. Three heterozygotes with high HbF programs had elevated circulating fetal hemoglobin levels (5% to 7%), and two of these individuals had substantially elevated, albeit unexplained, reticulocyte counts. These observations suggest that whereas many Saudi β^s heterozygotes have high HbF programs and have by these criteria a form of nondeletion HPFH, the mutation is silent in the peripheral blood unless the rate of erythropoiesis is either transiently or chronically increased. This occurs when the increased HbF program is present in the setting of homozygosity for a β^s gene.

Relationship of patient to parent hemoglobin F program. The quantity of hemoglobin F produced in BFU-E–derived erythroblasts from Saudi sickle cell anemia patients, their parents, and normal American controls is displayed in Table 3. Though Saudi AS parents produce half as much HbF as their SS offspring, their HbF programs are significantly higher than normal Americans, demonstrating their inherent capacity for increased fetal hemoglobin production.

Figure 3 further demonstrates the relationship of the parental HbF program to the patient HbF program determined from the assays of HbF in BFU-E–derived erythroblasts. The results gathered from analysis of 15 families are shown. The SCA patient in each family is graphed in ascending order of HbF program and the same data for each patient's parents are shown. The mean ± 2 standard deviations for normal American donors is also shown. Three patients had HbF programs that were within or only slightly higher than the normal Caucasian range, as did their parents who were available for study. The remaining patients had

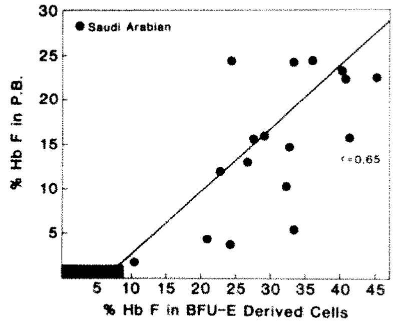

Fig 1. Percent HbF in peripheral blood versus percent HbF in BFU-E-derived cells in 27 Saudi patients (●) with sickle cell disease. Mean ± 2 SD for normal American donors is shown in the shaded area. $r = 0.65$, $P < 0.05$.

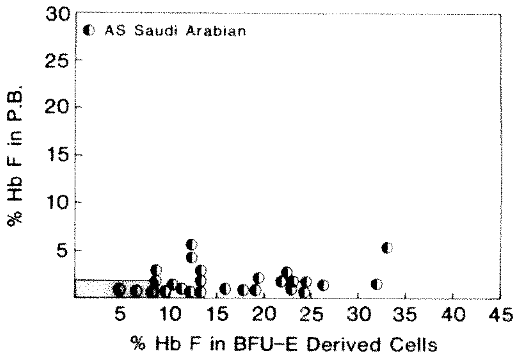

Fig 2. Percent HbF in peripheral blood versus percent HbF in BFU-E-derived cells for 27 Saudi patients (O) with sickle cell trait. Mean ± 2 SD for normal American donors is shown in the boxed area.

Table 3. Assessment of HbF in Saudi BFU-E–Derived Erythroblasts

Genotype	No.	% HbF in P.B.	% HbF in BFU-E–Derived Cells
Saudi SS	17	17.4 ± 9.3	30.4 ± 8.6*
Saudi AS	27	1.2 ± 1.07	16.1 ± 6.4†
American Normal	8	<2.0	6.1 ± 2.1

The % P.B. HbF represents the percent of HbF in peripheral blood measured by alkali denaturation. Normal range in CHMC hematology lab less than 2%. The % HbF in BFU-E–derived cells was measured by radioimmunoassay. Measurements indicate mean ± 1 SD.

*$P < 0.001$ when compared to Saudi AS.

†$P < 0.001$ when compared to American normal.

moderately to markedly elevated HbF programs. In every case, at least one of their parents had an HbF program well above the normal range. Analysis of patient versus mean parental HbF program revealed a significant ($P < 0.05$) positive correlation ($r = 0.51$).

Characterization of the β-globin gene complex by DNA analysis. Five DNA restriction site polymorphisms in the 5′ haplotype of the globin gene cluster were studied. These included the Hinc II site 5′ to the ϵ gene, Hind III in the IVS-2 of $G\gamma$ and $A\gamma$-globin gene, and Hinc II sites in the $\psi\beta1$-globin gene and 3′ to it. In several patients, all of the five intended sites could not be analyzed. Eighteen Saudi sickle cell anemia patients were studied. Ten of these with high HbF programs were clearly homozygous for the 5′ haplotype ++−++. Six others, patients who also had increased HbF programs, had results consistent with homozygosity for this haplotype although one to two sites were not assayed due to insufficient DNA. Due to the strong association of the +− Hind III combination with the ++−++ haplotype of the β^s chromosome in Saudi sickle cell anemia, any chromosome with the +− Hind III sites in gamma regions was considered to bear the entire ++−++ haplotype when the other sites were unknown. Two high HbF patients were heterozygous for the haplotype.

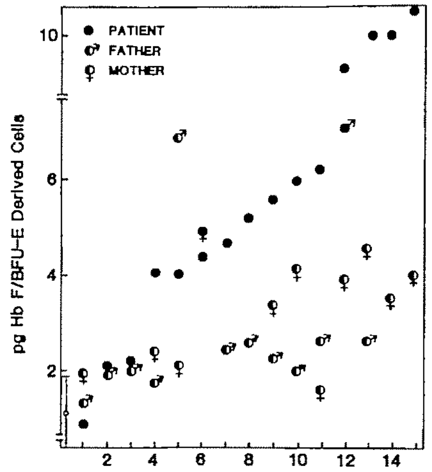

Fig 3. Relationship of parental HbF program to patient HbF program in Saudi Arabian sickle cell disease. Fifteen families are shown. The sickle cell patient (●) in each family is graphed in ascending order of pg hemoglobin F/BFU-E–derived cell. The pg hemoglobin F/BFU-E–derived cell for each parent with sickle cell trait (O) or sickle cell disease (●) is shown with symbols to distinguish mother and father. The mean ± 2 SD for normal American donors is shown on the lower left.

Material suitable for assay of restriction enzyme polymorphisms was available from only one of the three patients with low fetal hemoglobin. Of great interest was the fact that this patient was homozygous for the haplotype −++−− rather than the common ++−++ seen in the patients with high HbF programs.

To evaluate further the association of the ++−++ haplotype with the HbF program, we analyzed the latter in individuals both homozygous and heterozygous for the haplotype and with different beta-globin genotypes. The results are shown in Table 4. Nearly all individuals either homozygous or heterozygous for the ++−++ haplotype and homozygous for the β^s gene have markedly elevated HbF programs. That homozygosity for β^s has no influence on the HbF program unless β^s is associated with at least one chromosome with the ++−++ haplotype is suggested by the single relevant case. Homozygosity for the ++−++ haplotype does not itself control the level of the HbF program expressed in BFU-E–derived erythroblasts. This is demonstrated by the fact that individuals homozygous for the ++−++ haplotype but heterozygous for β^s had only modest elevations of HbF programs. Further studies will be required to explain this complicated relationship.

DISCUSSION

Sickle cell anemia patients from the eastern province of Saudi Arabia are unusual because of their high fetal hemoglobin levels, the relative mildness of their clinical disease, and the fact that heterozygotes do not ordinarily exhibit high fetal hemoglobin levels.[1-3,17,18] This distinguishes them from individuals heterozygous for the usual deletion or nondeletion forms of hereditary persistence of fetal hemoglobin and renders analysis of the inheritance of the defect difficult to perform.

In an effort to determine whether the high circulating fetal

Table 4. Effect of Interaction of B^s With the ++−++ Haplotype on the HbF Program

B Globin Genotype	++−++ Haplotype	No. Haplotyped	% P.B. HbF	% HbF in BFU-E–Derived Cells
SS	Homozygous	12	19.1 ± 8.4	33.0 ± 7.3
	Heterozygous	2	17.9, 28.5	32.4, 40.2
	Null	1	2.1	10.4
AS	Homozygous	4	1.7 ± 1.4	17.3 ± 3.6
	Heterozygous	7	1.7 ± 1.5	15.5 ± 4.6
	Null	1	0.6	13.0

SS or sickle trait patients are grouped based on being homozygous, heterozygous, or null for the "++−++" haplotype in the ϵ through $\psi\beta1$-globin gene region of the β-globin gene cluster. Due to the strong association of the +−Hind III combination with the ++−++ haplotype, any chromosome with the +−Hind III sites in gamma regions was considered to bear the entire ++−++ haplotype when other sites were unknown. The % P.B. HbF represents the percent of HbF in peripheral blood measured by alkali denaturation. The HbF program is represented by % HbF in BFU-E–derived erythroblasts. Results are expressed as mean ± 1 SD.

hemoglobin observed in Saudis with sickle cell anemis is an inherited characteristic, we measured the hemoglobins produced in erythroblasts derived from erythroid progenitors in the circulating blood of the Saudi patients, and their heterozygous parents. This method gives insight into the potential for HbF production in a population of progenitor-derived cells. This potential represents the product of the two independent mechanisms that regulate the circulating fetal hemoglobin level: the HbF-produced per F cell and the number of F cells produced per progenitor cell.[6] The latter cannot be accurately measured due to assay insensitivity that prevents detection of HbF in single cells unless it is present to an extent greater than 3 pg of F per cell (G. Dover, personal communication). Therefore, for the purposes of this assay, we assume that all of the erythroblasts found in progenitor-derived erythroid colonies produced in vitro are F cells. The HbF program estimated by this in vitro assay is of total F production, without loss of any population due to selective survival and without admixture of this erythroblast population with cells removed by multiple asymmetric divisions from their progenitors. This is important because present data strongly suggest that the HbF program, while clearly expressed in erythroblasts proximally derived from progenitors, is progressively diminished as erythroblasts pass through progressive rounds of asymmetric mitosis.[22-26]

Saudi sickle cell anemia patients produce increased levels of fetal hemoglobin in culture, compared to normal American controls.[6] The variance of the progenitor-regulated HbF program partially explains the variance of the circulating fetal hemoglobin of Saudi sickle cell patients but other factors including selective survival of erythrocytes containing fetal hemoglobin have a significant role.[3] That the increased HbF program of Saudi sickle cell anemia patients is highly variable suggests that the gene is highly regulated. In this respect, it differs from many of the known causes of HPFH in which the levels of HbF in peripheral blood of heterozygotes is quite constant. Indeed, the only constant finding with respect to the elevation of HbF in these patients is the regularly detected increased ratio of Gα to Aα globin. In the Saudi patients, Gγ represents 76.5 ± 5.1% of total HbF. In contrast, the Gγ to Aγ ratio in sickle cell anemia patients of African origin is variable but usually low.[21]

An important aspect of the data presented here concerns the detection of a variant of nondeletion HPFH in at least half of the β^s heterozygous parents of Saudi sickle cell patients with high circulating fetal hemoglobin. An increased level of circulating HbF is in most cases barely detectable in them, though the potential for high HbF production is found in the progenitors of many. The only heterozygotes with increased circulating fetal hemoglobin also had reticulocytosis. We hypothesize, therefore, that expression of the high HbF program may require erythropoietic stress such as the sickle cell anemia present in their children. The increased erythropoietin secretion secondary to anemia rapidly forces the generation of erythroblasts directly from relatively immature progenitor cells,[23] many of which produce erythroblasts with elevated HbF expression.[24,26] This model predicts that the actual amount of HbF produced in the erythroblasts derived from these progenitors depends upon two factors; the state of maturation of the progenitors (perhaps with respect to the number of quantal mitoses[25] completed in vivo prior to the culture) and the level of permissiveness of the gamma-globin gene.

Rank order analysis of patients and parental HbF programs shown in Fig 3 demonstrate an important relationship. Above an HbF program of 2.2 pg per cell, there was a significant correlation between the HbF program detected in the progenitors of the patients with that of at least one of their parents. These data provide strong evidence that the high fetal hemoglobin in Saudi sickle cell disease is inherited. These data plus those presented in Table 3 suggest but do not prove that, in Saudis, the high HbF program may be linked to the beta-globin gene cluster of chromosome 11.

To begin to examine the molecular basis of the increased HbF program in Saudis, we ascertained the haplotype of the epsilon through $\psi\beta 1$ region of chromosome 11 using restriction enzyme site polymorphisms. The limited haplotype data demonstrated that the "+ + − + +" 5′ haplotype is extremely common in these Saudis with sickle cell anemia and high fetal hemoglobin. This haplotype has been associated with Swiss HPFH,[12] and when linked to β^s has been associated with markedly raised fetal hemoglobin levels in Saudi Arabians,[12] and in a Jamaican family of mixed Asian Indian/African ancestory.[14] The haplotype "− + − + +" is one of the most common haplotypes on normal chromosomes.[27] Common to the two haplotypes are the four 3′ sites "+ − + +" and, where studied, atypically high levels of Gγ.[12,28] Also associated with some samples with this haplotype is a thymine for cytosine substitution at position −158 5′ to the Gγ gene,[29] which could itself increase the efficiency of the Gγ promotor, or merely represent an irrelevant but linked DNA polymorphism. Even if the −158 C → T substitution or another mutation in the + + − + + haplotype is responsible for increased Gγ/Aγ ratio, another regulatory system, perhaps akin to the recently reported −202 mutation 5′ to Gγ[30] or even separable from the β-globin gene cluster[31] may be responsible for the high total HbF production. The Saudi mutation is probably different from the −202 HPFH mutation because it appears to require anemic stress for expression. Cloning and sequencing of the γ gene flanking regions is necessary to determine all regions of difference between haplotypes. Systems for the study of the fetal to adult globin switch will also be necessary for full understanding of the relevance of point mutations detected.

Finally, though sickle cell disease in the eastern province of Saudi Arabia appears to be mild, and the combination of HPFH with sickle cell disease thought to ameliorate the latter,[32,33] the elevated HbF that characterizes these patients may not be solely responsible for their more benign clinical status. G6PD deficiency is common. Almost half of this population has microcytosis due to alpha thalassemia.[19] Alpha thalassemia is unlikely to be responsible for the elevated HbF in Saudis since patients with alpha thalassemia and sickle cell disease have lower levels of HbF than nonthalassemic sickle cell patients.[34] Although neither alpha thalassemia nor increased HbF, themselves, necessarily reduce the clinical severity of sickle cell disease,[35,36] the combination of both may be important. Only a field study will clarify that important issue. Clearly, continued studies of sickle cell anemia in the eastern province of Saudi Arabia will provide important information about the molecular biology of fetal hemoglobin production and the interacting factors affecting clinical severity.

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REFERENCES

1. Perrine RP, Brown MJ, Clegg JB, Weatherall DJ, May A: Benign sickle-cell anemia. Lancet ii:1163, 1972

2. Pembrey ME, Wood WG, Weatherall DJ, Perrine RP: Fetal haemoglobin production and the sickle gene in the oases of Eastern Saudi Arabia. Br J Haematol 40:415, 1978

3. Wood WG, Pembrey ME, Serjeant GR, Perrine RP, Weatherall DJ: HbF synthesis in sickle cell anemia: A comparison of Saudi Arab cases with those of African origin. Br J Haematol 45:431, 1980

4. Wrightstone RN, Huisman TJ: On the levels of hemoglobins F and A2 in sickle cell anemia and some related disorders. Am J Clinical Path 61:375, 1974

5. Serjeant GR: Fetal haemoglobin in homozygous sickle cell disease. Clinics in Hematol 4:109, 1975

6. Dover GJ, Boyer SH, Charache S, Heintzelman K: Individual variation in the production and survival of F cells in sickle cell disease. N Engl J Med 299:1428, 1978

7. Weatherall DJ, Clegg JB, Wood WG: A model for the persistence or reactivation of fetal hemoglobin production. Lancet ii:660, 1976

8. Javid J, Pettis PK, Miller JE: Radio-ligand immunoassay for human hemoglobin variants. J Immunol Methods 41:247, 1981

9. Friedman AD, Linch DC, Miller B, Lipton JM, Javid J, Nathan DG: Determination of the hemoglobin F program in human progenitor derived erythroid cells. J Clin Invest 75:1359, 1985

10. Nagel RL, Fabry ME, Pagnier J, Zohoun I, Wajcman H, Baudin V, Labie D: Hematologically and genetically distinct form of sickle cell anemia in Africa. N Engl J Med 312:880, 1985

11. Gilman JG, Huisman THJ: Two independent genetic factors in the B-globin gene cluster are associated with high $^G\gamma$ levels in the HbF of SS patients. Blood 64:452, 1984

12. Gilman JG, Harano T, Nakatsuji T, Bakioglu I, Reese AL, Gardiner MB, Huisman THJ: The ratio of the $^G\gamma$ and $^A\gamma$ chains: Variations due to anomalies at the molecular level. Ann NY Acad Sci 445:235, 1985

13. Old JM, Ayyub H, Wood WG, Clegg JB, Weatherall DJ: Linkage analysis of nondeletion hereditary persistence of fetal hemoglobin. Science 215:981, 1982

14. Wainscoat JS, Thein SL, Higgs DR, Bell JI, Weatherall DJ, Al-Awamy BH, Serjeant GR: A genetic marker for elevated levels of hemoglobin F in homozygous sickle cell disease? Br J Hematol 60:261, 985

15. Rovera G, Magarian C, Boun TW: Resolution of hemoglobin subunits by electrophoresis in acid urea polyarylamide gels containing Triton X-100. Anal Biochem 85:506, 1978

16. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

17. Perrine RP, John P, Pembrey M, Perrine S: Sickle cell disease in Saudi Arabs in early childhood. Arch Dis Child 56:187, 1981

18. Mallough A, Burke GM, Salamah M, Ahmed MS: Splenic function in Saudi children with sickle cell disease. Ann Trop Med 4:87, 1984

19. Pembrey ME, Weatherall DJ, Clegg JB, Bunch C, Perrine RP: Haemoglobin Bart's in Saudi Arabia. Br J Haematol 29:221, 1975

20. Pressler L, Higgs DR, Clegg JB, Perrine RP, Pembrey ME, Weatherall DJ: A new genetic basis for hemoglobin-H disease. N Engl J Med 303:1383, 1980

21. Gardiner MB, Reese AL, Headlee ME, Huisman THJ: The heterogeneity of the chain of fetal hemoglobin in HbS heterozygotes. Blood 60:513, 1982

22. Dover GJ, Boyer SH: Quantitation of hemoglobin within individual red cells: Asynchronous biosynthesis of fetal and adult hemoglobin during erythroid maturation in normal subjects. Blood 56:1082, 1980

23. Alpen EL, Cranmore D: Cellular kinetics and iron utilization in bone marrow as observed by Fe radioautography. Ann NY Acad Sci 77:753, 1959

24. Papayannopoulou TH, Kakomoto B, Buckley J, Kurachi S, Nute PE, Stamatoyannopoulos G: Erythroid progenitors circulating in the blood of adult individuals produce fetal hemoglobin in culture. Science 199:1349, 1978

25. Holtzer H, Biehl J, Antin P, Tokunaka S, Sasse J, Pacifici M, Holtzer S: Quantal and proliferative cell cycles: How lineages generate cell diversity and maintain fidelity, in Stamatoyannopoulos G, Nienhuis A (eds): Globin Gene Expression and Hematopoietic Differentiation. New York, Liss, 1983, p 213

26. Nathan DG: Regulation of erythropoiesis and hemoglobin production, in Stamatoyannopoulos G, Nienhuis A (eds): Globin Gene Expression and Hematopoietic Differentiation. 1983, p 365

27. Antonarkis SE, Boehm CD, Giardina PJV, Kazazian HH Jr: Nonrandom association of polymorphic restriction sites in the β-globin gene cluster. Proc Nat Acad Sci USA 79:137, 1982

28. Labie D, Pagnier J, Lapoumeroulie C, Rouobhi F, Dunda-Belkhodja O, Chardin P, Beldjord C, Wajcman H, Fabry ME, Nagel RL: Common haplotype dependency of high Gγ-globin expression and high HbF levels in β-thalassemia and sickle cell anemia patients. Proc Natl Acad Sci USA 82:2111, 1985

29. Gilman JG, Huisman THJ: DNA sequence variation associated with elevated fetal Gγ globin production. Blood 66:783, 1985

30. Collins FS, Stoeckert CJ, Serjeant GR, Forget BG, Weissman SM: $^G\gamma$ B+ Hereditary persistence of fetal hemoglobin: Cosmid cloning and identification of a specific mutation 5′ to the G gene. Proc Natl Acad Sci USA 81:4894, 1984

31. Boyer SH, Dover GJ, Sergeant GR, Smith KD, Antonarakis SE, Embury SH, Margolet L, Noyes R, Boyer ML, Bias WB: Production of F cells in sickle cell anemia: Regulation by a genetic locus or loci separate from the β-globin gene cluster. Blood 64:1053, 1984

32. Jacob GF, Raper AB: Hereditary persistence of foetal hemoglobin production and its interactions with the sickle cell trait. Br J Haematol 4:138, 1958

33. Stamatoyannopoulos G, Wood WG, Papayannopoulou TH, Nute PE: A new form of hereditary persistence of fetal hemoglobin in blacks and its association with sickle cell trait. Blood 46:683, 1975

34. Noguchi CT, Dover GJ, Rodgers GP, Serjeant GR, Antonarakis SE, Anagnou NP, Higgs DR, Weatherall DJ, Schechter AN: Alpha thalassemia changes erythrocyte heterogeneity in sickle cell disease. JCI 75:1632, 1985

35. Steinberg MH, Rosenstock W, Coleman MB, Adams JG, Platica O, Cedeno M, Rieder RF, Wilson JT, Milner P, West S, and the Cooperative study of sickle cell disease: Effects of thalassemia and microcytosis on the hematologic and vasoocclusive severity of sickle cell anemia. Blood 63:1353, 1984

36. Powars DR, Weiss JN, Chan LS, Schroeder WR: Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? Blood 63:921, 1984

Human Parvovirus B19-Induced Epidemic Acute Red Cell Aplasia in Patients With Hereditary Hemolytic Anemia

By Ulla M. Saarinen, Terence L. Chorba, Peter Tattersall, Neal S. Young, Larry J. Anderson, Erskine Palmer, and Peter F. Coccia

From March to August 1984, 26 patients with hereditary hemolytic anemia in northeastern Ohio developed acute, profound red cell aplasia. The patients included 14 males and 12 females 2 to 23 years old, with sickle cell anemia (20 cases), hemoglobin SC-disease (4 cases), sickle-beta-thalassemia (1 case), or hereditary spherocytosis (1 case). All had an acute onset of severe reticulocytopenia and anemia and prodromal symptoms of illness including fever, abdominal symptoms, headache, and arthralgias. Twenty-two received transfusions. Reticulocytosis occurred spontaneously within 2 to 14 days of presentation. In five acute-phase sera, 10^8 to 10^{12} viral particles/mL were detected by electron microscopy. Human parvovirus B19 DNA was demonstrated in high concentration by hybridization in the same five acute-phase sera and in low concentration in sera of eight additional patients. The five highly viremic sera inhibited erythroid colony formation in vitro. B19-specific IgM was detected in sera of 24/26 patients, and B19-specific IgG in 21 of 22 patients tested. Our results indicate that human parvovirus B19 was the etiologic agent in this large epidemic of life-threatening acute red cell aplasia in patients with hereditary hemolytic anemia.

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"APLASTIC CRISIS" in a patient with underlying hereditary hemolytic anemia is defined as a transient episode of pure red cell aplasia, with virtual absence of erythroid precursors in the bone marrow and with the absence of reticulocytes in the circulation. Because of the shortened red cell life span in hereditary hemolytic anemia, a temporary interruption of erythropoiesis leads to a precipitous fall in hematocrit and acute, life-threatening anemia. Recovery is heralded by a spontaneous, massive increase of erythropoiesis, also indicated by a burst of reticulocytes in the peripheral blood.[1–3]

The infectious nature of aplastic crisis in patients with hemolytic anemia has been suggested by reports both of family outbreaks and of clustering of attacks in time.[4,5] Epidemics have been reported from Jamaica, where outbreaks of aplastic crisis have been seen cyclically at about 5-year intervals.[6] The last Jamaican epidemic occurred during 1979–1980 with 38 identified cases.[6] A cluster of cases occurred simultaneously in England.[7,8] Although an infectious etiology was suspected when aplastic crisis in hereditary hemolytic anemia was first described,[1] it took over 30 years to obtain information about the causative agent. Recent studies have associated aplastic crisis with human parvovirus infection.[6–8]

During 1984 in northeastern Ohio we investigated a large epidemic of red cell aplasia involving 26 patients with hereditary hemolytic anemia. In this report we characterize the course of the illness and its pathogenesis, and present evidence indicating the etiologic role of human parvovirus B19 infection in the epidemic.

MATERIALS AND METHODS

Patients. In March 1984, three patients with sickle cell disease presented to Rainbow Babies and Childrens Hospital in Cleveland with profound anemia and reticulocytopenia. These cases prompted the development of a detailed, prospective study to investigate the etiology of aplastic crisis.

To identify as many cases of aplastic crisis as possible, we contacted all pediatric hematologists and most internist hematologists in northeastern Ohio, the American Sickle Cell Association in Cleveland, and all major health care providers for patients with sickle cell disease in Cleveland, as well as physicians with large black practices in Cuyahoga county.

Between March 13 and August 23, 1984, all known patients with hereditary hemolytic anemia and acute red cell aplasia admitted to hospitals in northeastern Ohio were prospectively enrolled in the study. During the last 4 months of 1984, no new cases appeared. Nineteen patients were admitted to Rainbow Babies and Childrens Hospital, Case Western Reserve University, Cleveland; one to Lakeside Hospital, the adult-care facility of the University Hospitals in Cleveland; one to St. Luke's Hospital, Cleveland; two to Kaiser-Permanente, Cleveland Heights and Parma; two to Akron Children's Hospital, Akron; and one to St. Joseph's Hospital, Lorain.

Case definition. A case of aplastic crisis was defined as a patient with underlying hereditary hemolytic anemia, who developed red cell aplasia of less than 2 weeks of duration, with a fall in the reticulocyte count to less than 50% of the patient's mean baseline value. The mean baseline reticulocyte count was the average value obtained from well-clinic visit records. Although most patients developed severe anemia requiring transfusion, hemoglobin or hematocrit values were not used as criteria for case definition.

Methods. A detailed history was taken, especially regarding preceding symptoms of illness. Additional information was obtained in most cases by telephone interview of the patient's mother or another adult family member. Physical examination was performed at the time of hospital admission and repeated daily until discharge. Peripheral blood counts, including hematocrit (Hct), hemoglobin (Hgb), reticulocyte count, platelet count, white blood cell count (WBC), and differential were measured during the hospitalization and the follow-up visits. The baseline levels of Hct and reticulocyte count were defined as the average of well-clinic visit records

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preferably in the previous 2 years, or when unavailable, from follow-up visits more than 2 months after hospital discharge. Venous blood was drawn during the acute and/or convalescent phase for virologic and serologic studies. The serum was stored at −70 °C until analyzed.

Laboratory methods. Acute-phase sera were evaluated for human parvovirus particles by negative-stain electron microscopy with uranyl acetate stain. B19 DNA was estimated in the sera by a modification of the DNA dot hybridization method.[9,10] In the hybridization procedure, aliquots corresponding to 0.5 μL of serum were digested with 100 μg/mL proteinase K for 30 minutes at 60 °C; an equal volume of 0.6N NaOH was then added, and the sample was hydrolyzed at 60 °C for another 30 minutes. An equal volume of 2 mol/L ammonium acetate (pH 7.0) was then added, and the sample was applied to pre-wet nitrocellulose filters with a slot-blotter apparatus (Schleicher and Schuell, Keene, NH). Slot blots were probed with high specific activity ^{32}P-labeled RNA, synthetized in vitro from a full-length copy of B19 DNA[11] inserted in the SP65 riboprobe vector. Probe synthesis and hybridization were performed as described by Zinn et al[10] and B19 DNA concentrations estimated by densitometric comparison with standards constructed by dilution, in negative serum, of purified 110S B19 virions,[11] with extinction coefficients derived for the minute virus of mice, another parvovirus.[12] The limit of detection was 0.30 ng/mL of serum. The viral DNA from acute-phase aplastic crisis sera was compared with prototype B19 DNA on alkaline agarose gels.[11] The virions from one case were purified and compared with other parvoviruses.[12]

The ability of viremic sera to inhibit erythroid colony formation in vitro was determined using normal bone marrow cells cultured in methylcellulose, by methods previously described.[13,14] Under the conditions of testing (dilutions of sera of 10^{-1} or greater, the absence of added complement, and adsorption at 4 °C) sera from normal individuals or those who have received multiple blood transfusions do not significantly inhibit erythroid or myeloid colony formation.

All sera were evaluated for B19-specific IgM and IgG antibodies in a capture enzyme-linked immunosorbent assay (ELISA) patterned after the radioimmunoassay method.[15] In the ELISA, goat antihuman IgG or IgM were used as capture antibody (Tago Laboratories, Burlingame, Calif) followed by the human serum sample, diluted 1:100, then B19 antigen from the acute-phase serum of an aplastic crisis patient, diluted 1:500, then a mouse monoclonal antibody (162-2B) against B19, and finally goat antimouse IgG H- and L-chain conjugated with peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md). The substrate was o-phenylendiamine (0.4 mg/mL) and hydrogen peroxide (0.015%) in citrate phosphate buffer.

Definition of reticulocyte count nadir. To compare the time course of illness among different patients, we selected the day of the lowest documented reticulocyte count, ie, the reticulocyte count nadir, as the reference point. This day was called "day 0," and subsequent days were called +1, +2, +3, etc, while the days preceding nadir were called −1, −2, −3, etc, respectively.

RESULTS

Subject description. During the 5½-month period beginning March 13 and ending August 23, 26 patients with underlying hereditary hemolytic anemia developed acute, profound red cell aplasia. The peak incidence occurred in April 1984. In contrast, during the preceding 3 years there had been no reported cases of red cell aplasia in patients with hemolytic anemia.

Fourteen patients were males and 12 were females. The patients were 2 to 23 years old (median 11 years), with incidence peaks of 3 and 14 years. The subjects included two sibling pairs and one mother-and-child pair. The time difference in the onset of illness as defined by the reticulocyte count nadir was 8 and 12 days in the two sibling pairs. The mother and child contracted the illness simultaneously.

The underlying hereditary hemolytic anemia was sickle cell disease (SS) in 20, hemoglobin SC disease (SC) in 4, sickle-beta-thalassemia (S-thal) in 1, and hereditary spherocytosis (HS) in 1 patient. The patient with HS was white; the other 25 were black.

Symptoms of illness. All patients had prodromal symptoms of illness for 1 to 17 days (median 4 days) prior to the manifestation of red cell aplasia. All patients reported fever, chills, lethargy, or malaise. Abdominal symptoms were reported by 85%: 58% had nausea or vomiting, 54% had abdominal pain, and 12% had diarrhea. Seventy-three percent suffered from a variety of aches and pains: 46% had headache, 27% had back pain, 27% had arthralgias, and 15% had myalgias or diffuse achiness. In addition, 42% had mild respiratory symptoms, consisting of rhinorrhea (23%), cough (19%), or conjunctivitis and photophobia (15%). Twenty-three percent had a skin rash. A skin eruption was observed in two patients during hospitalization. When questioned directly for dermatologic symptoms, four other patients reported having had a skin rash prior to hospital admission. The rashes had involved arms, face, legs, or trunk, and had been faint and maculopapular. The duration of rash was 3 to 21 days. All symptoms of illness resolved during hospitalization, usually within 3 to 5 days.

Red cell aplasia. The prominent hematologic feature was red cell aplasia, manifested as acute, severe anemia and profound reticulocytopenia (Fig 1). The nadir reticulocyte counts, which ranged from 0% to 2.2%, were below 1% in 85% (22/26) of the cases. The average baseline reticulocyte counts were 8.3% to 24.4% in SS and 3.2% to 5.8% in SC and S-thal patients (Fig 1). Severe anemia (Hct of below 20%) was documented in 23 of the 26 patients. The nadir hematocrits were 7% to 18% in SS (average baseline 19% to 28%) and 17% to 32% in SC and S-thal (average baseline 27% to

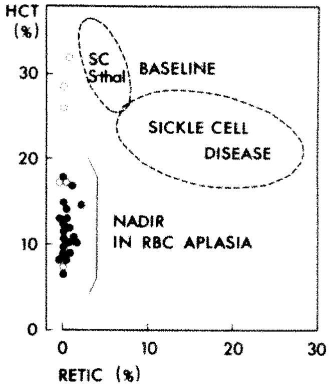

Fig 1. The nadir values of hematocrit and reticulocyte count in 26 patients with acute red cell aplasia. Solid circles: patients with sickle cell disease. Open circles: patients with other types of hemolytic anemia. The oval areas represent the baseline values of these patients.

34%). The patients with the three milder cases of anemia with nadir hematocrits of 26%, 28%, and 32% all had SC (Fig 1).

Bone marrow examinations performed in 3 patients 0 to 2 days after the reticulocyte count nadir revealed moderate to severe erythroid hypoplasia with few immature erythroid precursors. Cellularity and numbers of precursors in all other cell lines were normal. Erythropoiesis recovered rapidly in these patients with a reticulocytosis of 5% or more within 3 to 5 days.

The natural course of red cell aplasia is illustrated in one patient who refused blood transfusion on religious grounds (Fig 2). The life-threatening anemia with severe hypoxic symptoms was corrected by a sudden, spontaneous bone marrow recovery and a burst of nucleated red cells in the circulation, with reticulocyte count peaking at 44% (Fig 2).

Reticulocytosis occurred within 2 to 14 days of presentation and 2 to 8 days of the reticulocyte count nadir in all of the patients. In 5 patients we had the opportunity to document the true reticulocyte count nadir during the hospitalization by demonstrating an initial fall in the reticulocyte count. In all 5 patients, the duration of the reticulocytopenia from the true nadir to a reticulocyte count level of at least 2.5% was 6 to 8 days.

Platelets. No arrest of platelet production was demonstrated. The platelet count dropped below 150 × 10^9/L in only two patients. Marrow recovery was associated with thrombocytosis. Platelet count peaks over 500 × 10^9/L (range 509 to 1133 × 10^9/L) were seen in 13 patients, a median of day +4 from the reticulocyte count nadir.

White Blood Cells. Leukopenia usually did not occur. In contrast, many patients developed leukocytosis, in 9 patients exceeding 25 × 10^9/L and ranging up to 47.3 × 10^9/L (corrected for nucleated red cells) in association with early marrow recovery. A subgroup of patients presented with atypical lymphocytosis (over 10% in differential in 5/23), eosinophilia (over 5% in 9/23), a short-lived neutropenia (absolute neutrophil count below 1000 × 10^6/L in 4/23), and appearance of circulating plasma cells up to 5% (4/23). In three patients no differential white cell count was available. These changes in white blood cells tended to occur in the same individuals: 26% (6/23) had 2 to 4 of the abovementioned characteristics. The changes were short-lasting and were observed very close to the reticulocyte count nadir (days −1 to +1).

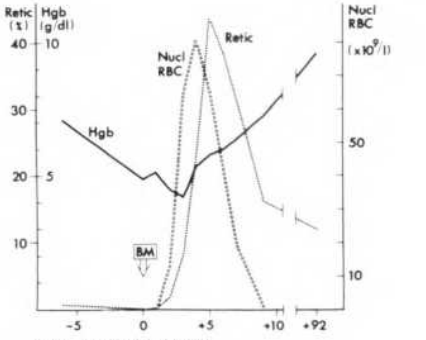

Fig 2. Natural course of aplastic crisis with sudden, spontaneous recovery in an 18-year-old patient who refused transfusion. Day 0 indicates reticulocyte count nadir. BM = bone marrow examination, in which the erythroid cell line was represented by only a few proerythroblasts.

Therapy in acute red cell aplasia. As treatment for the acute red cell aplasia and severe anemia, 22 of 26 patients received red cell transfusions; six were total or partial exchange transfusions. One patient refused blood transfusion on religious grounds (Fig 2), and 3 patients with hemoglobin SC disease did not require transfusion (Fig 1). In addition, the patients received intravenous fluids and symptomatic pain medication. Some were initially started on antibiotics because of suspected bacterial infection; however, no infections could be documented. The hospitalizations were in general short (median 4 days, range 2 to 13 days) and uneventful.

Virologic studies. Acute-phase sera were available for study in 21 patients. (In the remaining five cases the first serum sample was obtained at day +35 or later after the reticulocyte count nadir.) In five acute-phase sera, 10^8 to 10^{12} viral particles/mL were detected by electron microscopy (Fig 3). The purified virions of one acute-phase serum sample were found to contain 5.4 kb linear DNA species in 110 S particles physically indistinguishable from prototype B19 or other parvoviruses.[11,12]

B19-DNA, which was demonstrated by DNA hybridization, was detected in high concentration (>1000 ng/mL) in 7 acute-phase sera of 5 patients, and in low concentration (0.3 to 10 ng/mL) in the sera of an additional 8 patients (Fig 4). Serum samples with high-concentration B19-DNA and viral particles demonstrated by electron microscopy were drawn between day −5 to day +1 from the reticulocyte count nadir. Low-concentration antigenemia was detectable up to day +4 (Fig 4). In patients who were viremic, virus was also demonstrated in urine and throat gargle.[16]

Serologic studies. B19-specific IgM was detected in the sera of 24/26 patients. The highest titers were observed at

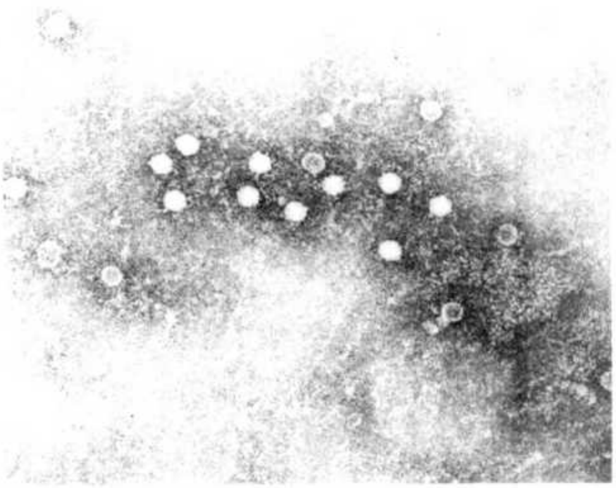

Fig 3. Aggregate of parvovirus-like particles in acute-phase serum from a patient with aplastic crisis. The particles are negatively stained with uranyl acetate. Original magnification × 156230; current magnification × 107,798.

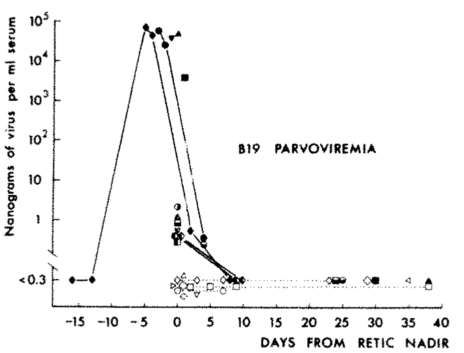

Fig 4. B19 parvoviremia, measured by the DNA hybridization technique, related to time from reticulocyte count nadir (= day 0). Individual patients with acute red cell aplasia are shown with different symbols. Solid symbols: patients with high-concentration viremia. Half-open symbols: patients with low-concentration viremia. Open symbols: patients in whom no viremia was found.

the reticulocyte count nadir and during the subsequent 10 days. The titers stayed at detectable levels for about 140 days (Fig 5). In six patients with viral antigenemia at the nadir, B19-specific IgM seroconversion was demonstrated.

B19-specific IgG was negative at the reticulocyte count nadir in all patients (Fig 5), but thereafter rose to high concentrations and tended to stay high for 4 to 5 months (Fig 5). The earliest positive specific IgG was detected on day +1 after the reticulocyte count nadir, and on day +2 after the true nadir. Follow-up was insufficient to determine the duration of IgG antibody positivity. B19-specific IgG was detected in 21 of the 22 patients with convalescent-phase sera available. Fourteen patients demonstrated a fourfold or greater rise in IgG antibody.

In 24 out of our 26 cases we had evidence of human parvovirus B19 infection; 24 had specific IgM detected, and 13 of these also had B19-DNA demonstrated in acute-phase serum. In two cases, no evidence for B19 infection could be obtained. Both of these patients, however, fit our criteria for aplastic crisis. For one patient, studied on day +1, convalescent-phase serum was not available. Sera from the other patient, obtained on days +1 and +29, were negative for virus and for specific IgM and IgG antibodies.

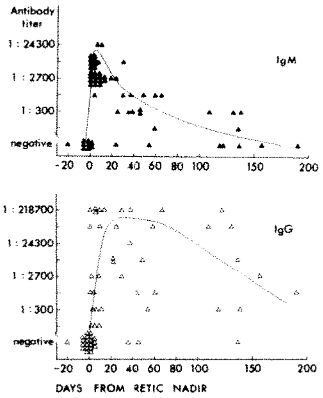

Fig 5. B19-specific IgM and IgG antibody titers related to the time from the reticulocyte count nadir (= day 0). Individual values of 26 patients (1 to 5 values per patient) are plotted, and the average courses are indicated by the curves (modified after linear regression lines over three times intervals per curve).

Inhibition of erythroid colony formation. The 5 acute-phase sera that demonstrated high-concentration B19-DNA and parvovirus particles on electron microscopy inhibited erythroid colony formation in vitro. The colony-forming units-erythroid (CFU-E)–derived colonies were completely inhibited at serum dilutions of 1:10 and 1:100 (Fig 6). The more primitive burst-forming units-erythroid (BFU-E)–derived colonies were inhibited completely at 1:10 and partially at 1:100 serum dilution (Fig 6). The sera retained strong inhibitory effects on CFU-E and BFU-E after being heated to 56 °C. Parvovirus-negative sera, screened blindly, were not inhibitory in the erythropoiesis assay. The parvovirus-negative acute sera were comparable to the positive ones regarding the previous blood transfusion status of the patients. The colony-forming units, granulocyte-macrophage (CFU-C)–derived colony growth was not affected by viremic sera.

DISCUSSION

This is the first large epidemic of pure red cell aplasia reported in the United States. In the preceding 3 years, no children with aplastic crisis were diagnosed in northeastern Ohio. Our series consisted of 24 children and 2 young adults. Two previous reports from the United States included 6 adult patients during 1980 to 1982 in Illinois[17] and 6 children during 1979 to 1983 in Washington, D.C.[18] Occurrence of aplastic crisis is thought to confer lasting immunity, because the illness usually affects children under 16 years of age,[6] and more than one episode during a lifetime is rare.[6,19]

Aplastic crises have been previously documented in a variety of hemolytic anemias, eg, sickle cell disease,[2,3,6-8,17-20] hereditary spherocytosis,[1,4,21] hereditary elliptocytosis,[22] thalassemia major,[23] thalassemia intermedia,[17] hemoglobin E/thalassemia,[24] pyruvate kinase deficiency,[25] paroxysmal noc-

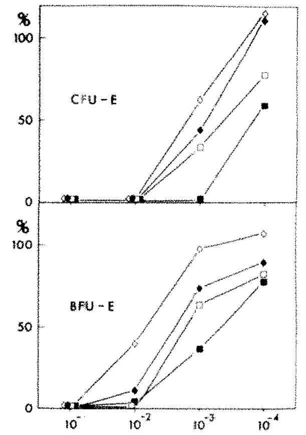

Fig 6. Inhibition of erythroid colony growth by human parvovirus B19. CFU-Es and BFU-Es were assayed in the presence of viremic sera, and measured as percentage of colony growth of the control. Serum dilution curves of four patients (indicated by different symbols) with the highest viremia peaks (over 40,000 ng of virus per mL serum) are shown.

turnal hemoglobinuria,[26,27] and acquired hemolytic anemias.[28-30] The underlying hereditary hemolytic anemias observed in our series were sickle cell disease, hemoglobin SC disease, sickle-beta-thalassemia, and hereditary spherocytosis. Although aplastic crisis is not associated with a certain type of hereditary hemolytic anemia, the severity of the aplastic crisis correlated with the severity of underlying hemolytic anemia; 3 of our 4 cases with hemoglobin SC disease did not require red cell transfusions (Fig 1).

The incubation period of epidemic red cell aplasia suggested in the literature has been 9 ± 3 days[4] or 17 days.[31] The incubation times observed in the two sibling pairs in our study were 8 and 12 days, in agreement with the shorter estimate.[4] The prodromal symptoms of illness included fever, pains and aches, abdominal symptoms, and mild respiratory symptoms. Associated skin rash was reported in 23%. A very similar pattern of prodromal symptoms has been described repeatedly since the first reports of aplastic crisis in hemolytic anemia.[1-4,8,17,18] A skin rash was rarely described in these reports. Because most of the patients were black, a rash might not have been easily detected; however, in Mortimer's review of spherocytosis in white patients, only one out of 64 had a skin rash.[4]

The prodromal symptoms were followed by a precipitous fall of hematocrit and detection of severe reticulocytopenia and anemia at hospital admission. The duration of profound reticulocytopenia was 6 to 8 days in the 5 cases in whom the true nadir of the reticulocyte count could be documented during the hospitalization. The literature offers detailed enough case reports of a few cases only,[1-3,18,19,25] but in those the total duration of the reticulocytopenia also was very consistently 6 to 7 days. The whole course of the illness, from the onset of prodromal symptoms to the reappearance of circulating reticulocytes, is about 10 to 12 days.[4]

Interest has been focused on the red blood cell changes that occur during aplastic crisis, but little attention has been paid to the white blood cells or the platelets. It is generally thought that these cells are not affected. Slight thrombocytopenia[1,8,17,25] and slight leukopenia[17,19,25] have occasionally been observed. Although we did not see thrombocytopenia or leukopenia in general, a subgroup of patients presented with atypical lymphocytosis, eosinophilia, neutropenia, and circulating plasma cells. These characteristics tended to occur in the same individuals, and they also tended to occur very close to the true reticulocyte count nadir. There are occasional reports of atypical lymphocytes,[3] eosinophilia,[2] plasma cells,[1-3] or neutropenia[1,2,8] occurring during aplastic crisis, but usually not in combination. The white blood cell changes may be very short in duration, and because the true reticulocyte count nadir has usually passed when the patient is admitted to hospital, the characteristic white cell changes may have been missed. On the other hand, at the marrow recovery stage, the burst of erythropoiesis was preceded by leukocytosis and thrombocytosis in many of our patients.[1,19]

Evidence of human parvovirus B19 as the etiologic agent was obtained by demonstrating parvovirus-like particles by electron microscopy in 5 acute-phase sera, and by demonstrating B19-specific DNA by hybridization in high concentration in the same 5 acute-phase sera and in low concentration in the sera of 8 additional patients. The high-concentration viremia was observed during a very short period before and at the reticulocyte count nadir, ie, from day −5 to day +1 (Fig 4). The time of onset and the duration of viremia before the manifestation of aplastic crisis is not known. One of our cases with high-concentration viremia on days −5 and −4 had nonviremic serum samples available from days −16 and −13 (Fig 4). We cautiously conclude that viremia is unlikely to occur before the appearance of prodromal symptoms.

As further evidence of parvovirus B19 infection, B19-specific IgM was detected in sera of 24/26 patients, of whom 6 seroconverted. B19-specific IgM was detectable for about 140 days (Fig 5), compared to the 10-week estimate previously given.[32] B19-specific IgG was detectable in convalescent-phase sera of 21/22 patients, including 14 seroconversions. Specific IgG stayed detectable throughout the follow-up (Fig 5); IgG is known to be measurable for 6 months[15] or longer.[17] Based on these data, 24 of the 26 patients with epidemic red cell aplasia had evidence of B19 infection.

The five acute-phase viremia sera with viral particles seen by electron microscopy and B19-DNA in high concentration all inhibited erythroid colony growth in vitro. CFU-E–derived colonies appeared more sensitive to the inhibitory effect of viremic sera than the BFU-E–derived colonies. CFU-C–derived colonies were unaffected. These data are consistent with direct effect of the virus on erythroid progenitor cells in bone marrow as the pathogenetic mechanism in epidemic red cell aplasia. Partially purified parvovirus has been shown to inhibit proliferation of isolated erythroid progenitor cells of the CFU-E stage of development,[14] and parvovirus has been demonstrated in erythroid progenitor cells infected in vitro by specific immunofluorescence and electron microscopy.[33]

Human parvovirus B19 seems to be widespread throughout the world. B19-specific IgG has been detected in 25% of normal blood donors in France,[34] in over 40% in Australia,[35] and in 61% in England.[15] The effects of B19 do not appear to be restricted to bone marrow erythroid aplasia. Recent studies suggest that a mild acute exanthematous disease in children, called "fifth disease" or erythema infectiosum, is also caused by human parvovirus.[16,36,37] Fifth disease outbreaks have been recently reported from England,[36] Japan,[38,39] Finland,[40] and Manitoba, Canada.[41] Most interestingly, during our epidemic of red cell aplasia in 1984 in northeastern Ohio, an epidemic of fifth disease with over 450 reported cases occurred concurrently in the same geographic area.[16]

In vitro studies have shown that the autonomously replicating parvoviruses require their host cell to traverse the S-phase of the mitotic cycle for viral replication to proceed.[42] Thus parvoviruses would be expected to grow to high concentrations in rapidly replicating cells such as those of hyperplastic bone marrow or the tissues of the developing fetus. Indeed many parvoviruses have been shown to be teratogenic and fetocidal in their animal hosts.[42] For example, the porcine parvovirus is well recognized as a cause of reproductive failure due to intrauterine fetal death.[43] In this light it is of considerable interest that fetal infection with the B19 parvovirus has recently been associated with human hydrops fetalis[44] and late intrauterine fetal demise.[45] B19 virus has now also been reported in association with acute polyarthritis and prolonged arthropathy in adults.[46,47] In addition, speculation has recently been raised about a possible parvoviral etiology of rheumatoid arthritis.[48]

The present knowledge suggests that acute red cell aplasia in patients with underlying hereditary hemolytic anemia is probably the most serious and life-threatening illness caused by human parvovirus B19. Work must continue to elucidate the complete spectrum of human disease caused by this agent.

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REFERENCES

1. Owren PA: Congenital hemolytic jaundice. The pathogenesis of the "hemolytic crisis." Blood 3:231, 1948

2. Singer K, Motulsky AG, Wile SA: Aplastic crisis in sickle cell anemia. A study of its mechanism and its relationship to other types of hemolytic crises. J Lab Clin Med 35:721, 1950

3. MacIver JE, Parker-Williams EJ: The aplastic crisis in sickle-cell anemia. Lancet i:1086, 1961

4. Mortimer PP: Hypothesis: The aplastic crisis of hereditary spherocytosis is due to a single transmissable agent. J Clin Pathol 36:445, 1983

5. Editorial: Bone marrow aplasia and parvovirus. Lancet ii:21, 1983

6. Serjeant GR, Topley JM, Mason K, Serjeant BE, Pattison JR, Jones SE, Mohamed R: Outbreak of aplastic crises in sickle cell anemia associated with parvovirus-like agent. Lancet ii:595, 1981

7. Pattison JR, Jones SE, Hodgson J, Davis LR, White JM, Stroud CE, Murtaza L: Parvovirus infections and hypoplastic crisis in sickle-cell anemia. Lancet i:664, 1981

8. Anderson MJ, Davis LR, Hodgson J, Jones SE, Murtaza L, Pattison JR, Stroud CE, White JM: Occurrence of infection with a parvovirus-like agent in children with sickle cell anemia during a two-year period. J Clin Pathol 35:744, 1982

9. Kafatos FC, Jones CW, Efstratiadis A: Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucl Acids Res 7:1540, 1979

10. Zinn K, DiMaio D, Maniatis T: Identification of two distinct regulatory regions adjacent to the human beta-interferon gene. Cell 34:865, 1983

11. Cotmore SF, Tattersall P: Characterization and molecular cloning of a human parvovirus genome. Science 226:1161, 1984

12. Tattersall P, Cawte PJ, Shatkin AJ, Ward DC: Three structural polypeptides coded for by minute virus of mice, a parvovirus. J Virol 20:272, 1976

13. Mortimer PP, Humphries RK, Moore JG, Purcell RH, Young NS: A human parvovirus-like virus inhibits haematopoietic colony formation in vitro. Nature 302:426, 1983

14. Young NS, Mortimer PP, Moore JG, Humphries RK: Characterization of a virus that causes transient aplastic crisis. J Clin Invest 73:224, 1984

15. Cohen BJ, Mortimer PP, Pereira MS: Diagnostic assays with monoclonal antibodies for the human serum parvovirus-like virus (SPLV). J Hyg Camb 91:113, 1983

16. Chorba T, Coccia P, Holman RC, Tattersall P, Anderson LJ, Sudman J, Young NS, Kurczynski E, Saarinen UM, Moir R, Lawrence DN, Jason JM, Evatt B: Parvovirus B19 infection: The cause of epidemic aplastic crisis and erythema infectiosum (fifth disease). J Infect Dis (in press)

17. Rao KR, Anderson MJ, Hodgson J, Jones SE, Pattison JR: Infection with parvovirus-like virus and aplastic crisis in chronic hemolytic anemia. Ann Intern Med 98:930, 1983

18. Kelleher JF, Luban NLC, Cohen BJ, Mortimer PP: Human serum parvovirus as the cause of aplastic crisis in sickle cell disease. Am J Dis Child 138:401, 1984

19. Charney E, Miller G: Reticulocytopenia in sickle cell disease. Am J Dis Child 107:450, 1964

20. Mann JR, Cotter KP, Walker RA, Bird GWG, Stuart J: Anemic crisis in sickle cell disease. J Clin Pathol 28:341, 1975

21. Kelleher JF, Luban NLC, Mortimer PP, Kamimura T: Human serum "parvovirus": A specific cause of aplastic crisis in children with hereditary spherocytosis. J Pediatr 102:720, 1983

22. Grech JL, Cachia EA, Calleja F, Pullicino F: Hereditary elliptocytosis in two Maltese families. J Clin Pathol 14:365, 1961

23. Jandl JH, Greenberg MS: Bone-marrow failure due to relative nutritional deficiency in Cooley's hemolytic anemia. Painful "erythropoietic crises" in response to folic acid. N Engl J Med 260:461, 1959

24. Chatterjea JB: Haemoglobinopathy in India, in Jonxis JHP and Delafresnaye JF (eds): Abnormal Haemoglobins: A Symposium. Oxford, Blackwell Scientific Publications, 1959, p 322

25. Duncan JR, Potter CG, Cappellini MD, Kurtz JB, Anderson MJ, Weatherall DJ: Aplastic crisis due to parvovirus infection in pyruvate kinase deficiency. Lancet ii:14, 1983

26. Crosby WH: Paroxysmal nocturnal hemoglobinuria: Report of a case complicated by an aregenerative (aplastic) crisis. Ann Intern Med 39:1107, 1953

27. Chanarin I, Dacie JV, Mollin DL: Folic acid deficiency in haemolytic anaemia. Br J Haematol 5:245, 1959

28. Gasser C: Akute Erythroblastopenie: 10 Fälle aplastischer Erythroblastenkrisen mit Riesenproerythroblasten bei allergisch-toxischen Zustandsbildern. Helv Paediatr Acta 4:107, 1949

29. Miesch DC, Baxter MR, Levin WC: Acute erythroblastopenia. Pathogenesis, manifestations, and management. Arch Intern Med 99:461, 1957

30. Meyer LM, Bertcher RW: Acquired hemolytic anemia and transient erythroid hypoplasia of bone marrow. Am J Med 28:606, 1960

31. Anderson MJ: The emerging story of a human parvovirus-like agent. J Hyg Camb 89:1, 1982

32. Anderson MJ, Davis LR, Jones SE, Pattison JR, Serjeant GR: The development and use of an antibody capture radioimmunoassay for specific IgM to human parvovirus-like agent. J Hyg Camb 88:309, 1982

33. Young NS, Harrison M, Moore JG, Cohen B, Mortimer B, Humphries RK: Direct demonstration of the human parvovirus in erythroid progenitor cells infected in vitro. J Clin Invest 74:2024, 1984

34. Courouce AM, Ferchal F, Morinet F, Muller A, Drouet J, Soulier JP, Perol Y: Human parvovirus infections in France. Lancet i:160, 1984

35. Shneerson JM, Mortimer PP, Vandervelde EM: Febrile illness due to a parvovirus. Br Med J 280:1580, 1980

36. Anderson MJ, Jones SE, Fischer-Hoch SP, Lewis E, Hall SM, Bartlett CLR, Cohen BJ, Mortimer PP, Pereira MS: Human parvovirus, the cause of erythema infectiosum (fifth disease)? Lancet i:1378, 1983

37. Anderson MJ, Higgins PG, Davis LR, Willman JS, Jones SE, Kidd IM, Pattison JR, Tyrrell DAJ: Experimental parvoviral infection in humans. J Inf Dis 152:257, 1985

38. Okabe N, Kobayashi S, Tatsuzawa O, Mortimer PP: Detection of antibodies to human parvovirus in erythema infectiosum (fifth disease). Arch Dis Child 59:1016, 1984

39. Nunoue T, Okochi K, Mortimer PP, Cohen BJ: Human parvovirus (B19) and erythema infectiosum. J Pediatr 107:38, 1985

40. Meurman O, Blomqvist M, Valkeakari T: Parvovirus—erythema infectiosumin ja anemiapotilaitten hypoplastisten kriisien aiheuttaja. Duodecim 100:1313, 1984

41. Plummer FA, Hammond GW, Forward K, Sekla L, Thompsom LM, Jones SE, Kidd IM, Anderson MJ: An erythema infectiosum-like illness caused by human parvovirus infection. N Engl J Med 313:74, 1985

42. Siegl G: Biology and pathogenicity of autonomous parvoviruses, in Berns KI (ed): The Parvoviruses. New York, Plenum Press, 1984, p 297

43. Mengeling WL, Paul PS, Brown TT: Transplacental infection and embryonic death following exposure to porcine parvovirus near the time of conception. Arch Virol 65:55, 1984

44. Brown T, Anand A, Ritchie LD, Clewley JP, Reid TMS: Intrauterine parvovirus infection associated with hydrops fetalis. Lancet ii:1033, 1984

45. Knott PD, Welply GAC, Anderson MJ: Serologically proved intrauterine infection with parvovirus. Br Med J 289:1660, 1984

46. Reid DM, Reid TMS, Brown T, Rennie JAN, Eastmond CJ: Human parvovirus-associated arthritis: A clinical and laboratory description. Lancet i:422, 1985

47. White DG, Woolf AD, Mortimer PP, Cohen BJ, Blake DR, Bacon PA: Human parvovirus arthropathy. Lancet i:419, 1985

48. Simpson RW, McGinty L, Simon L, Smith CA, Godzeski CW, Boyd RJ: Association of parvoviruses with rheumatoid arthritis of humans. Science 223:1425, 1984

Stromal Cell–Associated Erythropoiesis

By Schickwann Tsai, Colin A. Sieff, and David G. Nathan

A novel cover slip–transfer culture system was designed to study the functional roles of stromal cells in hemopoiesis, particularly erythropoiesis. Human bone marrow stromal cell colonies were allowed to develop on small glass cover slips in liquid medium. The cover slips, along with the stromal cell colonies and progenitors attached to them were then transferred to a new tissue culture dish and overlaid with methylcellulose culture medium. No exogenous colony-stimulating factors except erythropoietin were supplied. Large erythroid bursts, comprising multiple subcolonies, developed on the stromal cells. In order to determine if stromal fibroblasts together with erythropoietin and serum proteins could support erythroid development, human bone marrow cells depleted of monocytes, macrophages, and T lymphocytes were allowed to adhere to monolayers of a homogeneous fibroblastoid human stromal cell strain ST-1 grown on cover slips. The cover slips were then washed to remove nonadherent cells, transferred to a new culture dish, and overlaid with methylcellulose culture medium containing fetal calf serum and erythropoietin. In this modified system as well, primitive erythroid progenitors migrated extensively on and within the stroma to form huge colonies of hemoglobinized erythroblasts that proceeded to enucleate. Our results indicate that (1) ST-1 cells together with serum proteins and erythropoietin can support the development of large erythroid bursts; (2) erythroid progenitors and precursors adhere to and migrate on and within the extracellular matrix elaborated by ST-1 cells; (3) erythroid progenitors are more adherent to the ST-1 cells or the extracellular matrix than are the more mature cells and possibly the myeloid progenitors.

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UNDERSTANDING of hemopoiesis has been advanced recently through the use of two in vitro culture systems, a short-term colony assay in semisolid culture media and a long-term liquid culture. In short-term colony assays, development of hemopoietic progenitors into colonies of differentiated cells depends on an exogenous supply of hemopoietic growth factors such as erythropoietin, erythroid burst-promoting activity (BPA), and granulocyte-monocyte colony-stimulating activity (GM-CSA).[1,2] BPA and GM-CSA are usually supplied in the form of medium conditioned by T lymphocytes, monocytes, placenta, and various tumor cell lines. In long-term liquid culture of bone marrow, an adherent stromal layer consisting of fibroblasts, macrophages, endothelial cells, and extracellular matrix is a prerequisite for sustaining hemopoiesis over several months.[3,4] However, only granulopoiesis, monocytopoiesis, and megakaryocytopoiesis normally proceed to the stage of terminal differentiation.[5,6] The development of erythroid progenitors is usually arrested at the stage of the primitive burst-forming unit (BFU-E), even in the presence of an exogenous supply of burst-promoting activity and erythropoietin.[7,8] Although Dexter et al demonstrated red blood cell production in long-term cultures by addition of anemic mouse serum, the efficiency of erythropoiesis in such a system is low compared with that of granulopoiesis.[9] The nature of this block in erythroid development in long-term liquid culture remains unknown. It has been postulated that the contact of erythroid progenitors with stromal cells inhibit their differentiation.[10]

Recently, we have shown that medium conditioned by a fibroblastoid stromal cell strain established from a human fetal liver long-term culture contains factors capable of supporting the formation of erythroid and myeloid colonies in short-term colony assays in semisolid medium.[11] In this report, we address whether stromal cells themselves can directly support erythropoiesis to the stage of hemoglobinization. In order to demonstrate this direct support, progenitors were placed in close contact with the stromal cells comprising fibroblasts and macrophages or fibroblasts alone in a cover slip–transfer culture system.

This culture system, designed for studying erythropoiesis in close association with stromal cells, was conceived with the following assumptions: (1) Long-term liquid cultures lead to competition for essential nutrients and/or growth factors among large numbers of stromal cells, erythroid and myeloid progenitors, and mature granulocytes and monocytes; (2) In the later stages of erythroid development, the biosynthetic machinery of erythroid precursors is geared toward the production of red blood cell–specific proteins such as hemoglobin. Enzymes required for certain metabolic pathways such as the citric acid cycle may be synthesized at reduced rates. As a result, the developing erythroid precursors become fastidious in their nutritional requirement and more sensitive to metabolic waste, while myeloid cells survive well in adverse environments. The overall effect is the suppression of erythroid development in such a system. The unfavorable environment of long-term culture can be ameliorated by culturing a small number of progenitors in close contact with a small number of stromal cells in a large volume of culture medium. This is accomplished in the present study by transferring stromal cells with associated progenitors developing on a cover slip to a new tissue culture dish containing a large volume of methylcellulose culture medium. The results demonstrate that primitive erythroid progenitors can migrate, proliferate, differentiate, and enucleate on and within the stroma, which in this experiment consists of a mixture of fibroblasts and macrophages or fibroblasts alone. No exogenous hemopoietic growth factors except erythropoietin and serum proteins are required. The erythroid progenitors and precursors remain associated with the stromal layer throughout their differentiation.

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MATERIALS AND METHODS

Cover slip–transfer culture using human bone marrow cells. 5 to 10 mL normal human bone marrow was obtained by aspiration from healthy adult donors and collected into preservative-free heparin. The risks of the procedure were explained and informed consent was obtained under guidelines established by the Dana-Farber Cancer Institute. The bone marrow was diluted three-fold with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco, Grand Island, NY), layered over Ficoll Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden), and centrifuged at 400 x g for 40 minutes at 20 °C. The cells at the interface were collected, washed three times with cold IMDM, and resuspended in IMDM supplemented with 20% fetal calf serum (FCS) and penicillin/streptomycin (Gibco). The final cell density was 0.8×10^5 cells/mL.

Nine glass cover slips (12 x 12 mm) (Bradford Scientific, Inc, Epping, NH) were flamed and placed without overlapping on the bottom of a 60 mm tissue culture dish (Becton, Dickinson and Co, Cockeysville, Md). 4.0×10^5 cells in 5 mL IMDM supplemented with 20% FCS and penicillin/streptomycin were added to the dish and incubated at 37 °C, 5% CO_2 for 10 to 20 days with biweekly medium changes. Once small colonies of fibroblastoid stromal cells (50 or 100 cells) developed on the cover slips, the latter were transferred to a new tissue culture dish and washed vigorously with IMDM to remove nonadherent cells. Ideally, such washed cover slips contained one to three colonies of fibroblastoid stromal cells with 5 to 10 small round mononuclear cells (ie, potential progenitors) tightly attached to the fibroblastoid cells. Each cover slip was then transferred to an individual 35-mm tissue culture dish (Becton, Dickinson and Co), with the cells facing upward. Two mL of methylcellulose culture medium mixture containing 30% FCS (Gibco), 1% bovine serum albumin (Sigma, St. Louis, Mo), 10^{-4} mol/L mercaptoethanol (Sigma), 0.9% methylcellulose and erythropoietin (2 U/mL) (Terry Fox Laboratory, Vancouver, BC) were layered over the cover slip. No burst-promoting activity was detected in this erythropoietin preparation at the concentration used in this experiment. No other known colony-stimulating factors were supplied. The cultures were incubated in a high humidity incubator at 37 °C, 5% CO_2 for 10 to 30 days. Colonies developing on the cover slips were examined at sequential intervals with an inverted microscope.

Preparation of human bone marrow cells highly enriched for hemopoietic progenitors. Bone marrow mononuclear cells were collected from the interface after Ficoll-Hypaque gradient centrifugation as described above, washed, and resuspended in IMDM/20% FCS. Monocytes were removed by overnight adherence to 100-mm Lux culture dishes (Miles Laboratories, Naperville, Ill) at 37 °C. Nonadherent cells were removed, centrifuged, and incubated for 30 minutes at 4 °C with previously determined optimal concentrations of a panel of monoclonal antibodies directed against myeloid, erythroid, and lymphoid maturation antigens (Table I). After two washes in IMDM/2% FCS and one wash in PBS/5% FCS, the cells were resuspended in PBS/5% FCS and incubated for one hour at 4 °C on tissue culture dishes coated with rabbit antimouse immunoglobulin antibodies. The nonadherent marrow cells were removed with two gentle washes. 2% to 8% of the recovered cells were hemopoietic progenitors and over 99% of T lymphocytes and 90% of monocytes were depleted.[16]

Isolation of a fibroblastoid stromal cell strain from a human fetal liver long-term culture. Details of the isolation and characterization of a fibroblastoid stromal cell strain, ST-1, from a human fetal liver long-term culture have been described.[11] Briefly, hepatocyte-free mononuclear cells prepared from the liver of an abortus after legal termination of pregnancy were used to establish a long-term culture. A stromal cell strain, ST-1, was isolated from the stromal layer of this long-term culture after 6 weeks. The ST-1 cells are adherent, diploid, fibroblastoid in morphology, synthesize collagen type I and fibronectin, are nonphagocytic, myeloperoxidase-negative, nonspecific esterase-negative, and do not synthesize laminin or collagen type IV. ST-1 cells are nontransformed and have a limited life span. They were cultured in Dulbecco's Modified Eagle's Medium (DME) (Gibco) supplemented with 20% FCS.

Table 1. Monoclonal Antibodies Used to Fractionate Bone Marrow

Antibody	Specificity	Source and Reference
Leu 1	T lymphocyte	Becton Dickinson
Leu 5b	T lymphocyte, some natural killer (NK) cells	Becton Dickinson
Leu 9	T lymphocytes	Becton Dickinson
Leu 12	B lymphocytes	Becton Dickinson
TG1	Myeloid maturation	P. Beverley[12]
Leu M1	Monocytes, granulocytes	Becton Dickinson
Leu M3	Monocytes	Becton Dickinson
Mo 1	Monocytes, myeloid maturation, NK cells	J.D. Griffin[13]
My 8	Monocytes, myeloid maturation	J.D. Griffin[14]
LICR.LON.R18	Glycophorin A	P. Edwards[15]

Colony assay in semisolid culture medium. Marrow cells enriched for progenitors were suspended in methylcellulose culture medium (described above) and plated in triplicate in a 24-well tissue culture plate at 5×10^4 cell/mL. Mo conditioned medium (a gift from Dr D. Golde) was added at 5% (vol/vol). Alternatively, a five-fold concentrate of the medium conditioned by ST-1 cells was used at a final concentration of 10% (vol/vol). The preparation of ST-1 conditioned medium has been described.[11] Erythropoietin was added at a final concentration of 2 U/mL. Cultures were incubated at 37 °C in a high humidity incubator with 5% CO_2. Colonies were scored on day 14.

Cover slip–transfer culture using a preformed stromal cell monolayer and enriched bone marrow progenitors. 8×10^4 ST-1 cells of 18 to 21 passages were suspended in 5 mL of DME supplemented with 20% FCS and cultured in a 60-mm tissue culture dish containing 9 flamed cover slips (12 mm x 12 mm). The cells reached 60% to 80% confluency three to five days later. The medium was discarded and 4×10^5 enriched human bone marrow progenitors, suspended in 5 mL IMDM supplemented with 20% FCS, were added to the ST-1 cover slip cultures. The cultures were incubated at 37 °C, 5% CO_2 for 2 hours or longer. The cover slips were then removed, placed in a new culture dish, and rinsed gently with IMDM to remove nonadherent cells. Usually, only 100 to 400 marrow cells remained attached to the ST-1 monolayer on each cover slip after rinsing. Each cover slip was then transferred individually to a 35-mm tissue culture dish. Two mL of methylcellulose culture medium mixture with 2 units of erythropoietin/mL were layered gently over the coverslip. No other exogenous colony-stimulating factors were added. The cultures were incubated at 37 °C in a high humidity incubator with 5% CO_2. Colonies were scored on days 10 to 16.

Immunofluorescence staining of cover slip–transfer cultures. When colonies had fully developed in the cover slip–transfer cultures, 2 mL of 8% formaldehyde in phosphate buffered saline (PBS, pH 7.4) were added to each 35-mm dish. One hour later, the medium and the fixative were discarded. The culture was rinsed three times with PBS. The cover slip was then overlaid with 15 uL of a mouse monoclonal antiglycophorin A antibody (a gift from Dr P. Edwards) of appropriate dilution and incubated at 37 °C for 30 minutes in a humidity chamber. The cover slip was then rinsed once with PBS, overlaid with 14 uL of rhodamine-conjugated goat antimouse immunoglobulins and incubated at 37 °C for 30 minutes. The cover slip was then rinsed sequentially with PBS and distilled water and mounted in a mixture of glycerol and gelatin on a glass slide. Immunofluorescence microscopy was carried out with a Zeiss microscope equipped with epifluorescence.

Dimethoxybenzidine staining of cover slip–transfer cultures. After fixation as described above, the cover slip was rinsed three times with PBS and distilled water and air dried. One mL of 3,3'-dimethyoxybenzidine solution (1% in 70% methanol) (Sigma) was added to each dish. Two minutes later the dimethoxybenzidine solution was decanted. One mL of hydrogen peroxide solution (2% in 64% ethanol) was added for two minutes. The cover slip was then rinsed with distilled water, counterstained with hematoxylin for 30 seconds, rinsed with tap water, air dried, and mounted in Permount (Sigma) on a glass slide.

RESULTS

Bone marrow stromal cell–associated erythropoiesis in cover slip–transfer culture. After cover slips to which bone marrow stromal cells and progenitors had adhered were washed and transferred into a new tissue culture dish containing 2 mL of methycellulose culture medium and erythropoietin, the fibroblastoid stromal cells divided and migrated outward from the center of the small stromal cell colony. Normally, the stromal cell colony contained several macrophages. However, these macrophages could undergo only a few cell divisions. Therefore, in most cultures over 99% of the stromal cells were fibroblasts. Erythroid progenitors usually divided slowly in the beginning. Later, they multiplied faster and migrated extensively on the surface of the stromal fibroblasts (Fig 1A) and continued in this fashion for one to two weeks. In contrast to conventional short-term colony cultures, hemoglobinization was less synchronized from one subcolony to another; furthermore, the erythroid colonies developing in this culture system exhibited two unusual features: (1) the size of the colony was usually huge ($>10^4$ cells); (2) the number of subcolonies in each erythroid burst was great, sometimes over 20. Each subcolony was in intimate contact with the stromal cells (Fig 1B). Immunofluorescence staining using a mouse monoclonal antiglycophorin A antibody revealed that all cells in such subcolonies were strongly positive for glycophorin A (Figs 1C, 1D). Occasionally, macrophages were found

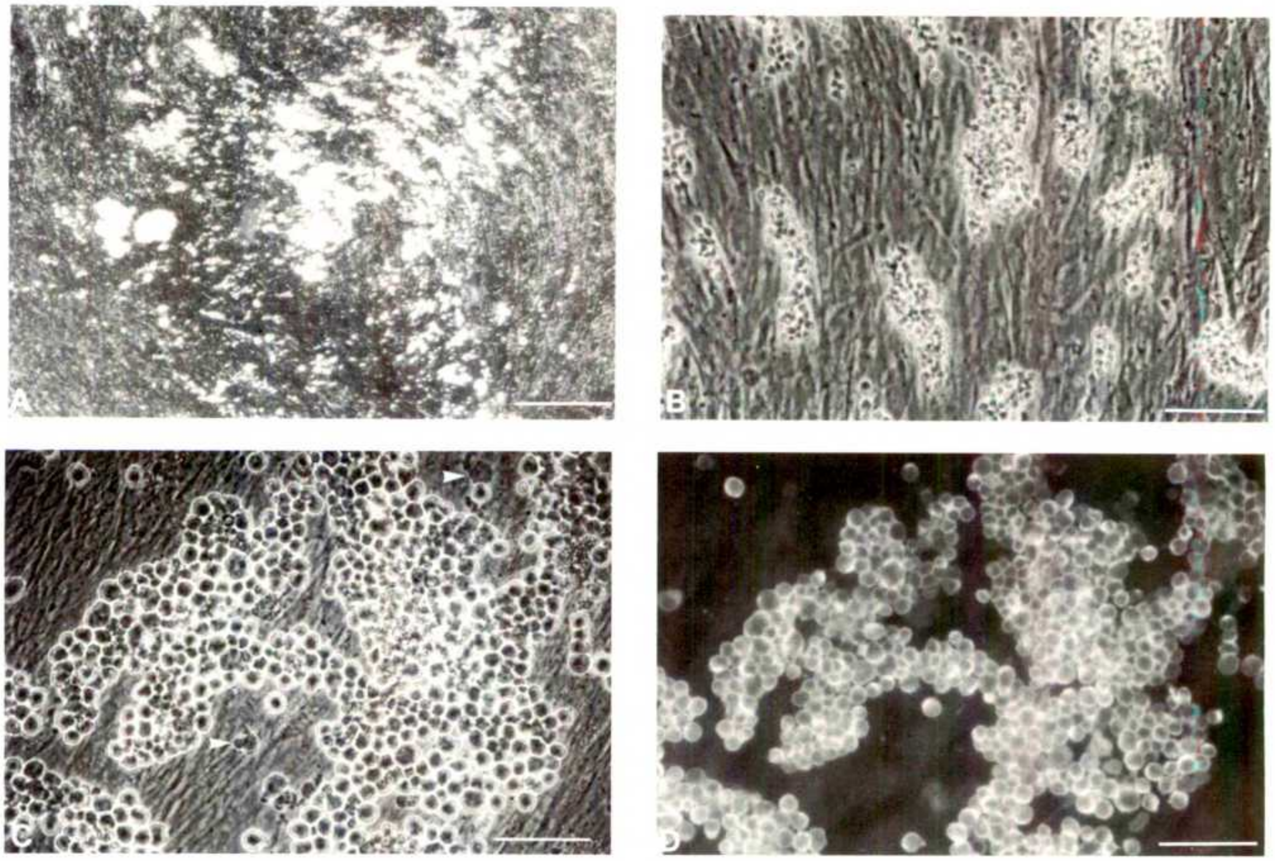

Fig 1. A typical erythroid burst colony developed in the cover slip–transfer culture. (A) Lateral illumination was used to highlight the erythroid colony which has a white swirling pattern. The fibroblasts in the background are only barely visible as gray streaks due to lateral illumination (Bar = 500 u). (B) A higher magnification photomicrograph of several subcolonies on the periphery of the colony shown in A. Each subcolony is intimately associated with the fibroblasts underneath. The latter are relatively free of other cell types. The tight, aggregated appearance of the individual subcolonies is typical of erythroid colonies after hemoglobinization. All subcolonies were orange-colored when this picture was taken. Phase contrast (Bar = 100 u). (C) An even higher magnification of a small portion of the colony shown in A. A few macrophages are visible (arrowheads). Variation in size is characteristic of erythroblasts at the beginning of hemoglobinization. Phase contrast (Bar = 50 u). (D) An immunofluorescent micrograph of the part of the colony shown in C. The colony was labeled with a monoclonal antibody specific for glycophorin A, followed by rhodamine-conjugated goat antimouse immunoglobulin. All cells except the few macrophages are strongly positive for glycophorin A which is specific for erythroid cells (Bar = 50 u).

around or in some subcolonies. However, "erythroblastic islands" consisting of a central macrophage and surrounding erythroblasts were never found.

Erythroid bursts can develop within the bone marrow stroma. Frequently, erythroid progenitors (probably BFU-Es) migrated from the surface of the stroma into the stroma and proliferated within the stroma to form honeycomb-like colonies. (Fig 2A–D). Occasionally, the progenitors emerged again from within the stroma and migrated extensively on the surface of the stromal cells (Fig 2C–D). At the time of hemoglobinization, the progenitors stopped migrating and proliferated to form individual subcolonies. Upon hemoglobinization, the cells became smaller and the whole subcolony appeared to contract to form tight clusters.

Mixed erythroblast-granulocyte-monocyte colonies can develop in the cover slip–transfer cultures. Mixed colonies containing erythroblasts, granulocytes, and monocytes could develop in the cover slip–transfer culture system, although no exogenous burst-promoting activity (BPA) or multicolony stimulating factor was included in the culture medium (Fig 3A & B). This finding extended our previous observation that the fibroblastoid stromal cells derived from fetal liver secrete factors capable of supporting the development of CFU-GEM, BFU-E, CFU-GM, CFU-G, and CFU-M.[11]

Enucleation of erythroblasts occurs in the cover slip–transfer culture. After hemoglobinization, erythroblasts proceeded to enucleate. This process is best observed in colonies embedded in stroma or colonies sandwiched between the cover slip and the bottom of the culture dish (Fig 4A, B). In the preenucleation stage, the nuclei became smaller and denser and were pushed to one side of the cytoplasm (Fig 4A). The nuclei were expelled along with some perinuclear material. The membrane resealed and the cells gradually assumed a biconcave shape typical of red blood cells (Fig 4B). Erythroblasts that developed on the surface of the stromal cells also enucleated, but this was less easily visualized.

Fibroblasts together with serum proteins and erythropoietin can support the development of large erythroid bursts. The experiments described above showed that bone marrow stromal cells could support the proliferation, differentiation, and hemoglobinization of erythroid progenitors. No exogenous colony-stimulating activity except erythropoietin was required. Presumably the stromal cells supplied the growth factors required by the progenitors. In addition, the stromal cells provided the extracellular matrix in which the progenitors migrated. Although most of the stromal cells were fibroblasts, small numbers of macrophages were always

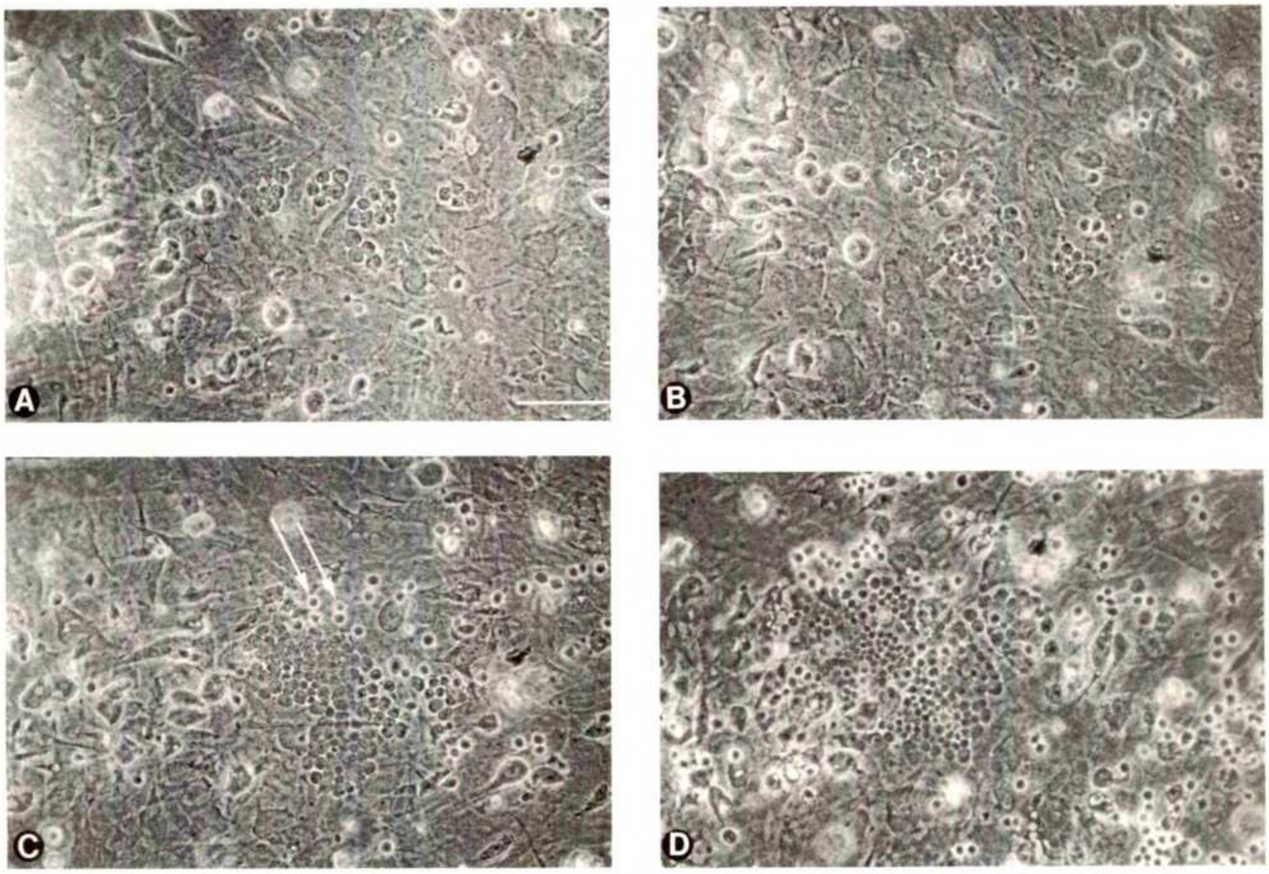

Fig 2. Sequential phase contrast photomicrographs of an erythroid colony embedded in the stroma. (A) Day 13. The erythroid progenitors appear here as five groups (center field) of phase-dense blasts with prominent nucleoli and very little cytoplasm. The stroma is a mixture of fibroblasts and macrophages (Bar = 100 u). (B) Day 14. Cell number has doubled. (C) Day 15. The groups have enlarged and coalesced. Note some progenitors migrating outward across the stroma (arrows). (D) Day 18. Note the migrating progenitors surrounding the honeycomb-like colony. Hemoglobinization began on day 20.

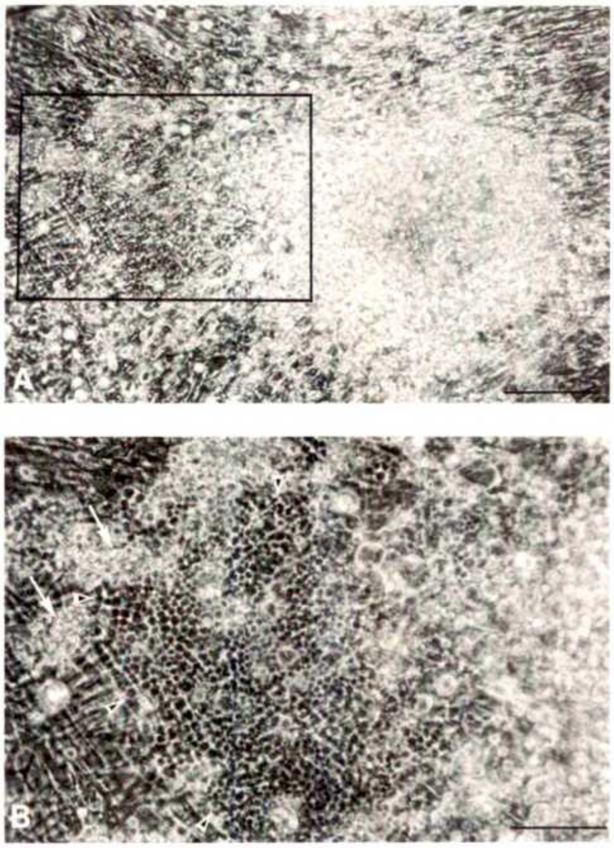

Fig 3. (A) A mixed colony containing granulocytes, monocytes, and erythrocytes. The granulocyte/monocyte portion is located on the right side and erythroblasts on the left side. They are embedded in the thick stroma and cannot be seen at this magnification (Bar = 500 u). (B) A magnified view of the boxed area in A. The erythroblasts appear here as the honeycomb-like colony outlined by arrowheads. A few erythroid clusters (arrows) are also seen in the left upper corner. The larger cells on the right side are monocytes and granulocytes. Phase contrast (Bar = 200 u).

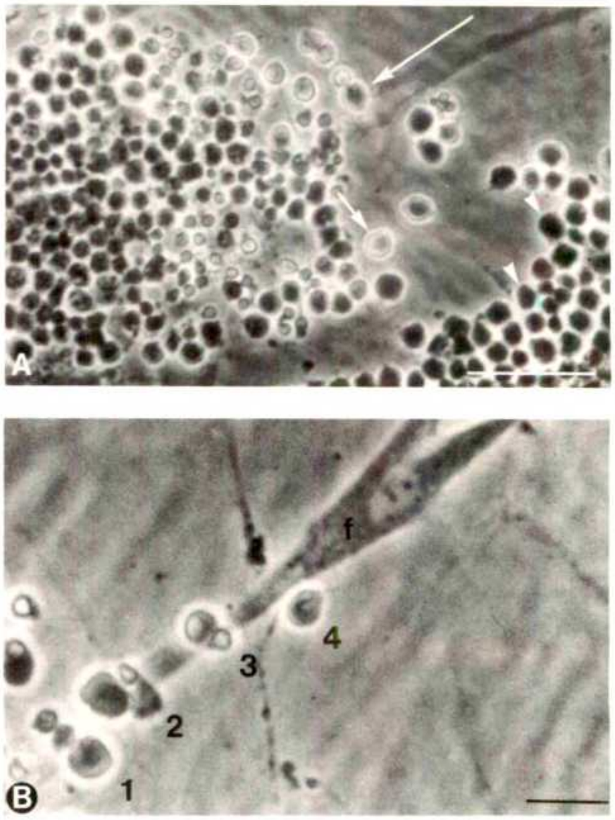

Fig 4. (A) An erythroid colony sandwiched between the cover slip and the bottom of the culture dish was photographed at the time of enucleation. All round cells in this field are erythroid. Erythroblasts (arrowheads); Reticulocyte (short arrow). A cell in the process of enucleation (long arrow) (Bar = 50 u). (B) Four cells at different stages of enucleation. 1 & 2: Erythroblasts in the process of expelling their nuclei and part of their cytoplasm. 3: Enucleation is completed. 4: A biconcave, enucleated reticulocyte. f = fibroblast. Phase contrast (Bar = 20 u).

detected. In order to determine whether fibroblasts alone could support erythropoiesis, we developed a modified cover slip–transfer culture system using enriched human marrow progenitors depleted of monocytes/macrophages and T-lymphocytes. The frequency of different progenitors in such enriched preparations is shown in Table 2. The enriched progenitors were allowed to adhere to a monolayer of ST-1 cells grown on a cover slip. The nonadherent marrow cells were then washed away gently. The cover slip, along with the adherent marrow progenitors, was transferred to a new culture dish and overlaid with methylcellulose culture medium containing erythropoietin (2 U/mL) and incubated. The progenitors migrated on or within the preformed ST-1 monolayer and formed large colonies. A typical erythroid colony developed in such a system is shown in Fig 5A–C. As evident in Fig 5B and C, the culture was virtually free of macrophages, yet erythropoiesis took place very efficiently. The same results were obtained when the addition of erythropoietin was delayed for three days.

Although it is impossible to exclude the presence of a few

Table 2. Frequency and Ratio of Progenitors in the Enriched Bone Marrow Cells

Source of CSA	BFU-E	CFU-C	CFU-GEM*	Frequency of Progenitors (%)	BFU-E:CFU-C Ratio
	(per 10^5 cells; mean ± SE, n = 3)				
Mo CM 5%	930 ± 233	792 ± 93	31	1.75	1.17
ST-1 CM† 10%	1146 ± 175	1031 ± 109	69	2.25	1.11
IMDM	338 ± 53‡	0	0	0.34	—

*Number is too small. Standard error is not calculated.

†Seven-day conditioned medium of ST-1 cells was dialyzed, lyophilized, and reconstituted with distilled water to give a 5-fold concentrate and used at a final concentration of 10% (vol/vol).

‡The colonies were small sized.

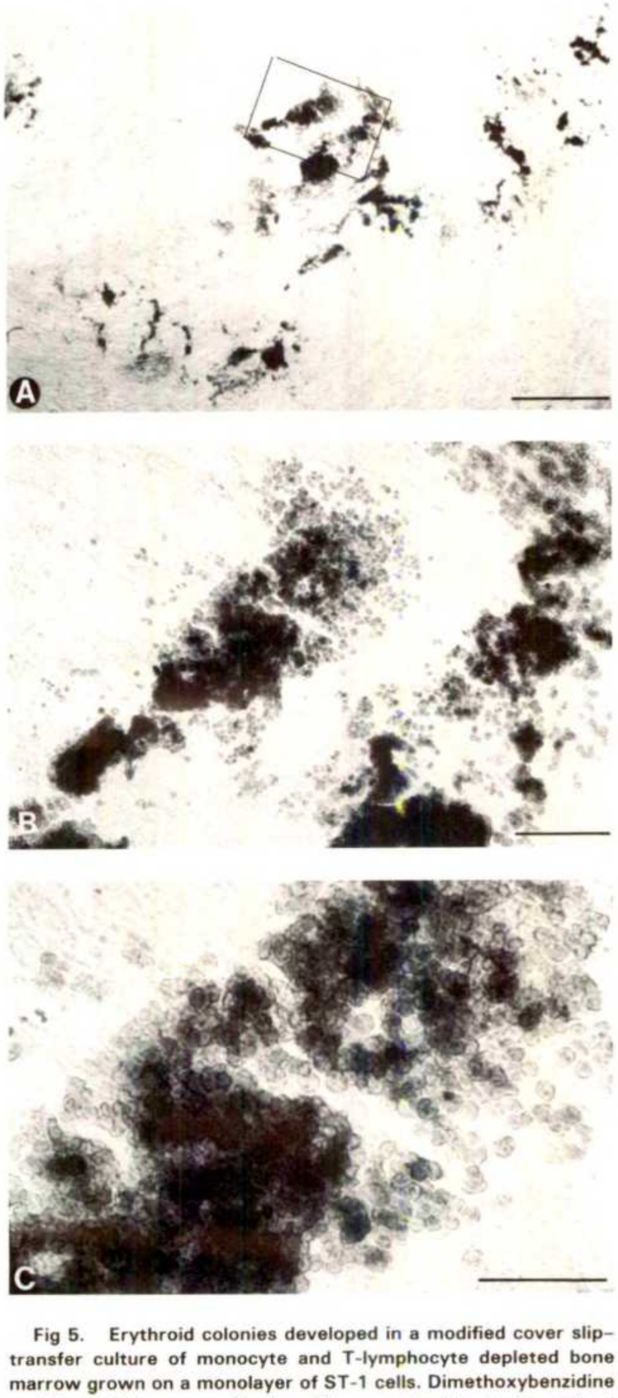

Fig 5. Erythroid colonies developed in a modified cover slip–transfer culture of monocyte and T-lymphocyte depleted bone marrow grown on a monolayer of ST-1 cells. Dimethoxybenzidine staining. (A) Low magnification. Three erythroid colonies stained intensely with dimethoxybenzidine are seen in this picture. The lacy structure in the background is the fibroblast population (Bar = 500 u). (B) Higher magnification of the boxed area in A (Bar = 100 u). (C) Even higher magnification of A. Note that the stroma immediately surrounding the colony is free of macrophages. Bright field microscopy (Bar = 50 u).

contaminating cells such as macrophages, the number of such contaminating cells, estimated to be less than 40 per 1.44 cm^2 cover slip surface area, is probably too small to contribute substantially to the burst promotion in this setting. In addition, the ST-1 cell–conditioned medium has been shown to have burst-promoting activity.[11] Therefore, we conclude that the ST-1 cells are responsible for the burst promotion in this system.

Primitive erythroid progenitors are more adherent to fibroblasts than nonprogenitors. Table 3 shows the results of an experiment in which enriched progenitors were incubated for varying periods of time on ST-1 cells before washing. The frequency of progenitors in the input population was about 2% (Table 2). Although only 100 to 400 cells adhered to each cover slip after only 2-hour exposure to the ST-1 cells, a high proportion (20%) was capable of colony formation. The majority of these colonies (90%) was derived from BFU-E and only a small number of myeloid colonies were observed (ratio 10 to 20:1). Longer periods of incubation did not result in greater progenitor cell adherence.

Dimethoxybenzidine staining reveals many erythroid colonies embedded within the fibroblast stroma. When cultures (using enriched progenitors and preformed ST-1 stromal layers) were fixed and stained with dimethoxybenzidine and hydrogen peroxide, many erythroid colonies previously obscured by the thick stromal layer were easily identified as patches of erythroblasts sandwiched between the stromal fibroblasts and the cover slip (Fig 6). An examination of four randomly selected cover slips stained with dimethoxybenzidine showed that more than half of the colonies had at least one subcolony embedded in the stroma. About 15% of the colonies were completely embedded within the stroma. Only 20% of the colonies were completely located on the surface of the stromal layer (Fig 7).

DISCUSSION

The survival and proliferation of murine multipotent hemopoietic stem cells can be maintained for several months in long-term cultures. In similar human cultures, hemopoiesis persists for only a few weeks. In both cases, only mature granulocytes, monocytes, and, to a lesser extent, megakaryocytes, are produced.[5,6] The differentiation of erythroid progenitors is invariably blocked at the BFU-E stage.[7] The nature of this block in erythroid differentiation in vitro remains unknown and represents a major obstacle to the study of erythropoiesis in vitro.

Based on their earlier work with mechanically agitated murine marrow long-term cultures, Dexter et al speculated that contact between CFU-E and the bone marrow stromal matrix inhibited their further differentiation.[10] Later, the same group reported that the addition of anemic mouse serum to murine long-term marrow cultures obviated the requirement for agitation to facilitate erythroid differentiation. It was speculated that there exists in anemic mouse serum a "second factor" in addition to erythropoietin that promotes erythroid development. It is clear, however, that erythropoiesis even in anemic mouse serum–stimulated long-term cultures is rather inefficient compared with granulopoiesis in the same system.

Our present study was stimulated by our earlier studies on long-term liquid cultures established from human fetal livers. In the second trimester, fetal liver hemopoiesis is still predominantly erythroid.[17] Very few granulocytes can be found. However, when fetal liver cells depleted of hepato-

Table 3. Type, Frequency, and Ratio of Colonies Developed in the Cover Slip–Transfer Culture Using Preformed ST-1 Monolayers and Enriched Bone Marrow Cells

Duration of Co-Culture Hour	No. of Adherent Cells per 10 cm^2	BFU-E	CFU-C	CFU-GEM	No. of Progenitors per 10 Adherent Cells	BFU-E:CFU-C Ratio
		(per 10 cm^2)				
2	1320	266	12	4	21.4	22.2
12	1525	229	10	7	16.1	22.9
24	1295	170	15	6	14.7	11.3
36	1970	234	11	6	12.7	21.3

*4 × 10^5 enriched human bone marrow progenitors suspended in 5 mL IMDM containing 20% FCS were cocultured for 2–36 hr with ST-1 monolayers grown on 12 × 12 mm cover slips placed on the bottom of a 60-mm dish. Marrow cells adhering to the ST-1 monolayer on the cover slips were counted after rinsing and transferring to a new culture dish at the end of the coculture periods. The marrow cells were small, round, and refractile and easily distinguished from the large, flat ST-1 cells.

†Six cover slips (12 mm × 12 mm) were used in each group. Data were normalized to give numbers of cells or colonies per 10 cm^2 cover slip surface area.

cytes were used to establish long-term liquid cultures, erythroblasts and CFU-Es disappeared rapidly from the cultures. Concomitant with the disappearance of erythroid precursors, there was an impressive proliferation and maturation of granulocytes/monocytes progenitors.[18] To investigate the mechanism underlying such a dramatic switch from erythroid to myeloid production in human fetal liver long-term cultures, we isolated a homogeneous stromal cell strain, ST-1, from a human fetal liver long-term culture. The ST-1 cells are fibroblastoid and appear to be derived from adventitial reticular cells in hemopoietic tissue. A similar cell strain has been isolated from human bone marrow and is similar to ST-1 cells with respect to morphology, surface antigens, and cytochemistry (S. Tsai, unpublished data). Medium conditioned by ST-1 cells supported the development of human bone marrow and human fetal liver BFU-E–, CFU-GM–, CFU-G–, and CFU-M–derived colonies in short-term colony assay in semisolid medium. Since the ST-1 cell-conditioned medium could support the development of erythroid progenitors in the semisolid medium culture and these cells were present in large numbers in fetal liver long-term cultures, we attempted to stimulate erythropoiesis by adding erythropoietin to human fetal liver long-term culture. Despite repeated efforts, erythroid development remained blocked at the BFU-E stage in human fetal liver long-term culture even in the presence of erythropoietin.

A clue to the resolution of this puzzling phenomenon came from our observation that whenever the cellular density was high in short-term colony assays in semisolid medium, erythroid colonies failed to develop successfully whereas granulocytes/monocytes colonies seemed unaffected. Thus it appeared that when cultures were crowded with cells, erythroid development was suppressed. Presumably there was a high negative selective pressure on erythroid progenitors and precursors when nutrients were limited and/or when there was an accumulation of metabolic wastes. To avoid this negative selection pressure on the erythroid precursors, the culture systems was modified accordingly in our cover slip–transfer culture system.

Our present study provides evidence that the contact between erythroid progenitors and stromal fibroblasts or stromal matrix is not, as previously suggested, inhibitory. Instead, stromal cells seem to provide the extracellular matrix on which progenitors migrate extensively. Consistent with this view are the recent findings that reticulocytes adhere to fibronectin, a major glycoprotein of extracellular matrix[19] and that some erythroid progenitors are more

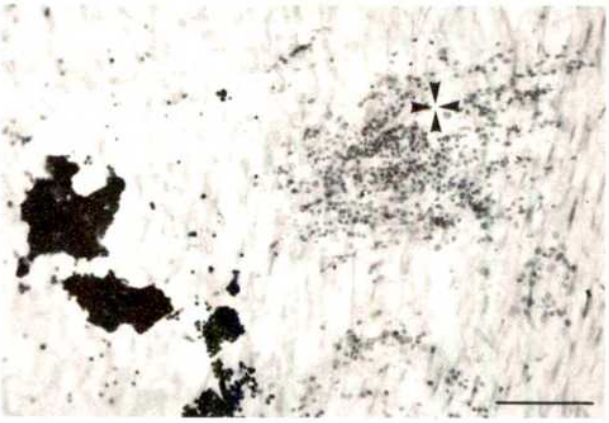

Fig 6. An erythroid colony (asterisk) embedded in the fibroblastoid stroma was revealed after staining with dimethoxybenzidine. Three subcolonies on the surface of the stroma are also seen here on the left side as intensely stained colonies. The fusiform structures are the nuclei of the fibroblasts. Bright field microscopy (Bar = 100 u).

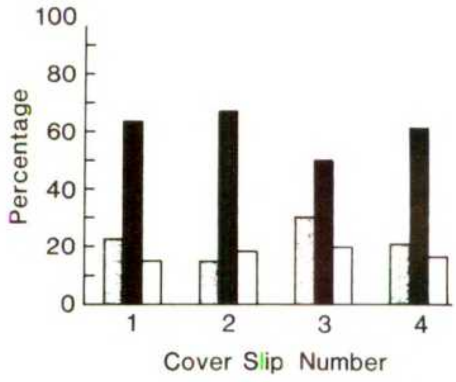

Fig 7. Classification of erythroid colonies on four randomly selected cover slips according to their location on or within the stroma: completely on the surface (shaded bar); partially embedded (solid bar); completely embedded (open bar).

effectively stimulated when placed in close contact with adherent stromal cells.[20] Indeed, it seems that erythroid progenitors have a tendency to migrate into extracellular matrix and establish colonies within it (Fig 7). In addition, stromal fibroblasts together with erythropoietin seem to provide sufficient regulators for the development of BFU-E–derived colonies. This is consistent with our previous finding that medium conditioned by a human stromal cell strain ST-1 could support the development of BFU-E–derived colonies in semisolid medium. Also important is the finding that erythropoiesis can proceed from progenitors to erythroblasts and on to enucleated reticulocytes in the absence of so-called "central macrophages."[21,22] The modified cover slip–transfer culture using human bone marrow cells depleted of monocytes, macrophages, and T-lymphocytes cultivated on monolayers of the fibroblastoid stromal cell strain demonstrated that erythroid development could proceed in the virtual absence of these putative accessory cells.

Among the marrow cells adherent to the ST-1 monolayers during a two-hour coculture period, approximately 20% are progenitors, representing a ten-fold increase over the frequency of progenitors in the input population (Tables 2, 3). This suggests that progenitors are probably more adherent to the stromal matrix or stromal fibroblasts than the more mature cells. Coulombel et al have reported that there are more CFU-GEM, BFU-E, or CFU-C in adherent layers than in nonadherent fractions of human bone marrow long-term cultures.[23] Also intriguing is the finding that over 90% of these progenitors were committed to erythroid development. Very few colonies were granulocytic. We are currently investigating the underlying mechanism of this phenomenon.

In summary, we have described a new culture system to facilitate the study of the functional roles of stromal cells and extracellular matrix in hemopoiesis. Like other physiologic processes in many organ systems, efficient hemopoiesis probably requires the interaction among many types of cells such as fibroblasts, macrophages, endothelial cells, and T cells. However, in order to gain insights into the interactions among different cell types, it is mandatory that purified, well-defined populations of cells be used in such analyses. Although the relevance of our finding needs to be substantiated in vivo, it seems that stromal fibroblasts, together with erythropoietin and serum proteins, could fulfill the minimal requirements for the development of erythroid burst colonies. Supporting this conclusion are the findings of Weiss and his colleagues in their electronmicroscopy studies of intact rat and human bone marrow: (1) hemopoietic cells appear immobilized in the marrow reticular meshwork; (2) in the initial phase (1 to 2 days) of recovery from irradiation, putative stem cells are seen closely apposed to the ramifying extended cytoplasmic processes of reticular cells[24]; (3) there is a "close and distinctive" relationship of reticular cells and erythroblasts, namely, the extensive apposition of the cytoplasm of reticular cells to maturing erythroblasts.[25] The cover slip–transfer culture system seems to duplicate all these conditions in vitro and is useful for studying the stromal cell-progenitor cell interaction. By adding purified populations of macrophages, endothelial cells, and T cells to this system, we can begin to analyze their influences on hemopoiesis.

REFERENCES

1. Bradley TR, Metcalf D: The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287, 1966
2. Pike BL, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76:77, 1970
3. Dexter TM, Wright EG, Krisza K, Lajtha LG: Regulation of hemopoietic stem cell proliferation in long term bone marrow cultures. Biomed 27:344, 1977
4. Dexter TM, Allen TD, Lajtha LG: Conditions controlling the proliferation of hemopoietic stem cells in vitro. J Cell Physiol 91:335, 1977
5. Williams N, Jackson H, Robellino EM: Proliferation and differentiation of normal granulopoietic cells in continuous bone marrow cultures. J Cell Physiol 93:435, 1977
6. Williams N, Jackson H, Sheridan APC, Murphy MJ, Elste A, Moore MAS: Regulation of megakaryopoiesis in long term murine bone marrow cultures. Blood 51:245, 1978
7. Testa NG, Dexter TM: Long-term production of erythroid precursor cells (BFU) in bone marrow cultures. Differentiation 9:193, 1977
8. Gregory CJ, Eaves AC: In vitro studies of erythropoietic progenitor cell differentiation, in Clarkson B, Marks PA, Till JE (eds): Differentation of Normal and Neoplastic Hematopoietic Cells. Cold Spring Harbor, NY, Cold Spring Harbor Publications, 1978, pp 179–192
9. Dexter TM, Testa NG, Allen TD, Rutherford T, Scolnick E: Molecular and cell biologic aspects of erythropoiesis in long-term bone marrow cultures. Blood 58:699, 1981
10. Eliason JF, Testa NG, Dexter TM: Erythropoietin-stimulated erythropoiesis in long-term bone marrow culture. Nature 281:382, 1979
11. Tsai S, Emerson SG, Sieff CA, Nathan DG: Isolation of a human stromal cell strain secreting hemopoietic growth factors. J Cell Physiol (in press)
12. Beverley PCL, Linch D, Delia D: Isolation of human haemopoietic progenitor cells using monoclonal antibodies. Nature 287:332, 1980
13. Todd RF, Nadler LM, Schlossman SF: Antigens on human monocytes identified by monoclonal antibodies. J Immunol 126:1435, 1981
14. Griffin JD, Ritz J, Nadler LM, Schlossman SF: Expression of myeloid differentiation antigens on normal and malignant cells. J Clin Invest 68:932, 1981
15. Edwards PAN: Monoclonal antibodies that bind to the human erythrocyte-membrane glycoproteins glycophorin A and band 3. Biochem Soc Trans 8:334, 1980
16. Sieff CA, Emerson SG, Mufson A, Gesner TG, Nathan DG: The dependence of highly enriched human bone marrow progenitors on hemopoietic growth factors and their response to recombinant erythropoietin. J Clin Invest 77:74, 1986
17. Hann IM, Bodger MP, Hoffbrand AW: Development of pluripotent progenitors in the human fetus. Blood 62:118, 1983
18. Cappelini MD, Potter CG, Wood WG: Long-term hemopoiesis in human fetal liver cell cultures. Brit J Haemat 57:61, 1984
19. Patel VP, Ciechanover A, Platt O, Lodish HF: Mammalian reticulocytes lose adhesion to fibronectin during maturation to erythrocytes. Proc Natl Acad Sci USA 82:440, 1985

20. Li CL, Johnson GR: Stimulation of multipotential, erythroid and other murine haematopoietic progenitor cells by adherent cell lines in the absence of detectable multi-CSF (IL-3). Nature 316:633, 1985

21. Bessis M: Living Blood Cells and Their Ultrastructure. Berlin, Springer-Verlag, 1973

22. Allen TD: Hemopoietic microenvironment in vitro: Ultrastructural aspects, in Porter R, Whelan J (eds): Microenvironments in Hemopoietic and Lymphoid Differentiation. Pitman, London, 1981, pp 38–67

23. Coulombel L, Kalousek DK, Eaves CJ, Gupta CM, Eaves AC: Long-term marrow culture reveals chromosomally normal hemopoietic progenitor cells in patients with Philadelphia chromosome-positive chronic myelogenous leukemia. N Engl J Med 308:1493, 1983

24. Weiss L: Hemopoiesis in mammalian bone marrow, in Porter R, Whelan J (eds): Microenvironments in Hemopoietic and Lymphoid Differentiation. Pitman, London, 1981, pp 5–21

25. Weiss L: The hematopoietic microenvironment of the bone marrow: An ultrastructural study of the stroma in rats. Anat Rec 186:161, 1976

Spontaneous Regression of a Monoclonal Proliferation of Large Granular Lymphocytes Associated With Reversal of Anemia and Neutropenia

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A 43-year-old male with a phenotypically homogeneous, expanded subset of T cells presented in 1981 with anemia and neutropenia. The surface antigen phenotype of 99% of the peripheral blood lymphocytes was T3+, T8+, T4−, and they were morphologically large granular lymphocytes (LGL). The same cells comprised 37% of the marrow nucleated cells. Eight months after he presented, the peripheral blood T8+, LGL diminished spontaneously, and the anemia and neutropenia completely resolved. The patient remains hematologically normal as of October 1984. To determine if the T8+, LGL represented a clonal expansion, DNA from peripheral blood lymphocytes collected and cryopreserved when the patient was neutropenic and anemic, and when he was hematologically normal, was analyzed for clonal T-cell antigen receptor gene rearrangements. Using Southern blot analysis, a clonal DNA rearrangement was demonstrated, and this clone diminished but was still demonstrable in peripheral blood lymphocytes collected in 1984. The above observations implicate the expanded T8+, LGL in the pathogenesis of the neutropenia and anemia, yet the exact mechanism remains to be elucidated.

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IN RECENT years a number of investigators have reported patients with neutropenia and/or anemia who have an increased number of peripheral blood and bone marrow large granular lymphocytes (LGL).[1-20] These lymphocytes usually express the T cytotoxic-suppressor phenotype (T8+ or Leu 2); however, in most of these cases they do not appear to suppress B-cell function.[2,4,8,13,18] They also have Fc receptors and express T3 antigen (Leu 4) but not T4 (Leu 3). These cells often have antibody-dependent cellular cytotoxicity,[2,3,5,7,8,12,18,19] and less frequently natural killer (NK) activity.[15,16,19] The possible role of these cells in the pathogenesis of the cytopenias remains to be elucidated. Until recently, it has not been possible to determine whether this expanded subset of lymphocytes represents an aberrant monoclonal or a reactive polyclonal proliferation.[20]

In B-cell proliferative disorders, the demonstration of monoclonal neoplasia has traditionally depended on the demonstration of the exclusive presence of one immunoglobulin light chain isotope (κ or λ). No comparable gene products are currently recognized on T lymphocytes, and the definitive proof of monoclonality of even phenotypically homogenous T-cell proliferations has been difficult. Immunoglobulin gene rearrangements are the earliest recognizable events in B-cell differentiation. Recently, specific immunoglobulin gene rearrangements as unique clonal markers have been demonstrated in some lymphomas and leukemias that were at a B-cell precursor stage before immunoglobulin gene products were being expressed.[21,22] The antigen-specific T-cell receptor has been identified and shown to be a polymorphic disulfide-linked heterodimer of 40–50 kd alpha and beta subunits.[23-25] cDNA clones encoding these chains have been isolated and expressed.[26-30] It is now recognized that these genes for the antigen receptor on T cells undergo rearrangements similar to those of immunoglobulin genes.[26-30] The availability of a cDNA probe which hybridizes with DNA encoding a constant portion of the β peptide of the T-cell antigen receptor has been used to demonstrate T-cell clonality in certain forms of T-cell leukemia.[31-40] The DNA rearrangements required to juxtapose the variable, diversity, and joining gene subsegments of the T_β gene together produce a change in the location of restriction endonuclease sites. A polyclonal population of normal T cells possesses numerous different T_β receptor gene rearrangements which result in multiple differently sized T_β receptor gene–containing DNA fragments after restriction enzyme digestion. When this collection of DNA fragments is analyzed on a Southern blot, no single rearranged band is detected because each rearrangement is below the threshold of sensitivity. In contrast, a monoclonal expansion representing a single cell's progeny will have a unique identifying DNA rearrangement pattern that can be detected on a Southern blot as a distinct band different in size from the band representing the germline (unrearranged) form of the gene.

We describe the use of a cDNA probe that hybridizes with the β chain of the T-cell antigen-receptor genes to demonstrate that the increased T8+, LGL in a patient with anemia and neutropenia are monoclonal. Of further interest, this clone spontaneously diminished, temporally associated with normalization of peripheral blood red cell and neutrophil counts.

CASE REPORT

On January 5, 1982 a 42-year-old male was referred to The Emory Clinic with a one-year history of pallor and a six-month history of easy fatigue and recurrent mouth ulcers. In November 1981, he first sought medical attention and was found to have a hematocrit of 23%, hemoglobin of 8.1 g/dL, a reticulocyte production index of 0.7, a MCV of 117 μm^3, a white blood count of 5900/μL, a neutrophil count of 413/μL, a lymphocyte count of 5074/μL, and a normal platelet count. The patient had an identical twin who was hematologically normal.

At the time of referral he had similar cytopenias. The majority of lymphocytes in the peripheral blood had abundant cytoplasm with azurophilic granules. On physical examination he had moderate

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mucosal pallor, was afebrile, and had no lymphadenopathy or hepatosplenomegaly. A bone marrow aspirate and biopsy revealed a hypercellular marrow with a lymphocytic infiltrate comprising 37% of the marrow cells. In the marrow differential there were 9% promyelocytes and 17% myelocytes, while more mature neutrophils totaled only 10%. There was a similar arrest in red cell precursor maturation with 6.5% pronormoblasts, 3% basophilic normoblasts, and only 5% rubricytes. Marrow cytogenetic analysis was normal. The peripheral blood lymphocytes formed E-rosettes (75%), and had IgG Fc receptors as determined by EA rosettes (78%). They also expressed the pan T antigen T3 (99%), suppressor/cytotoxic antigen T8 (99%).[12] Using peripheral blood lymphocytes cryopreserved in May 1982 (see below), we have recently demonstrated that these cells express the NK marker Leu 7 (77%),[41] but not the NK marker NKH1 (3%)[42] or the granulocyte-macrophage marker OKM1 (3%). As previously reported, the peripheral blood lymphocytes from this patient exhibit antibody-dependent cellular cytotoxicity, but not NK activity.[12]

Because of progressive anemia (hemoglobin 7.0 g/dL), the patient was transfused with packed red cells approximately every 3 to 4 weeks in February and March of 1982. On April 1, 1982 he was admitted to Emory University Hospital with a 3-day history of shaking chills, fever, and sinus congestion. He was found to have polymicrobial bacterial septicemia and x-ray evidence of sinusitis. Echocardiogram showed no vegetations on the heart valves. The hemoglobin on admission was 6.5 g/dL, the granulocyte count was 690/μL, and the lymphocyte count was 5,796/μL. He was treated with intravenous cephalosporin and animoglycoside for 3 weeks with clearing of the sinusitis and bacteremia. He also received six units of packed red blood cells during the 26-day hospitalization.

On May 11, 1982 he underwent leukapheresis to collect peripheral blood lymphocytes for cryopreservation. From May through July 1982, he received 10 units of packed red cells for persistent anemia. While receiving no medication, in August 1982 his hemoglobin stabilized without further transfusions and his neutrophil count increased to 1,209/μL. Subsequently, his blood counts gradually normalized and have remained so through November 1985.

MATERIALS AND METHODS

The patient was advised of procedures and attendant risks, in accordance with institutional guidelines, and gave informed consent. Light density peripheral blood lymphocytes and marrow cells for cell surface marker and DNA analysis were collected with EDTA anticoagulant and isolated by Ficoll-Hypaque density gradient centrifugation. The leukapheresis of 1×10^{10} peripheral blood lymphocytes on May 11, 1982 was accomplished using a continuous flow blood cell separator (IBM, Model 2997). The cells were separated on Ficoll-Hypaque and stored at −80 °C at 10^7 cells/mL with 10% dimethyl sulfoxide.

Sheep E-rosette and EA-rosette formation was assessed as previously described.[12] Cell surface antigens were detected using the following monoclonal antibodies: OKT3, OKT4, OKT8, OKM1 (Ortho Diagnostic Systems, Inc, Raritan, NJ), Leu 2, Leu 3, Leu 7 (Becton Dickinson Monoclonal Center, Inc, Mountain View, Calif), NKH1 (Coulter Immunology, Hialeah, Fla). Immunolabeled cells were quantified using indirect immunoperoxidase or avidin-peroxidase staining for light microscopy, or immunofluorescence for flow microfluorometry.

Marrow aspirates and biopsies for morphologic evaluation were obtained from the posterior superior iliac spine and processed as previously described.[12]

Analysis of T-cell antigen receptor gene rearrangements was performed as previously described.[39] The arrangement of T_β genes of the cryopreserved peripheral blood lymphocytes of the patient obtained during leukapheresis when the disease was active, and from pheripheral blood lymphocytes isolated from blood collected in October 1984 when the patient was in remission, was compared with the arrangement of these genes in peripheral blood buffy coat cells of normal individuals and in a B-cell line established from the patient using the Epstein-Barr virus (EBV). High molecular weight DNA samples were extracted from the cells and were digested to completion. The restriction enzyme *Bam*H1 DNA fragments were size fractionated over agarose gels by electrophoresis and transferred to nitrocellulose paper. Such paper-bound DNA fragments were hybridized to a random primed [^{32}P DNA] probe (86T5) to the constant domain of the T_β gene[26] obtained from Mark Davis. After washes at the appropriate stringency, the T_β configurations were visualized on autoradiograms.

RESULTS

Changes in peripheral blood cell counts with time. Serial peripheral blood counts and T-cell subset quantifications are displayed chronologically in Fig 1. These data demonstrate a striking temporal correlation between the decrease in the number of peripheral blood OKT8+ LGL, the increase in OKT4+ lymphocytes, and the normalization of the hemoglobin and neutrophil count.

Demonstration of the monoclonality of large granular lymphocytes. The arrangements of the T_β chain gene in normal buffy coat in polyclonal normal T cells in the patient's EBV-transformed B-cell line and the patient's peripheral blood mononuclear cells were examined by Southern analysis with a radiolabeled probe to the constant gene subsegment of this gene (Fig 2). The two T_β constant region genes were present on a single 24 kb *Bam*H1 fragment in all the peripheral white blood cell preparations (demonstrating the germline pattern of each of the 17 normal individuals examined). The T_β gene was also on a 24 kb *Bam*H1 fragment in the EBV-transformed B-cell line of the patient indicating that the germline pattern of this gene in this patient was comparable to that of other individuals. In polyclonal normal T cells, only the 24 kb *Bam*H1 band is evident since multiple, different sized, rearranged T_β gene-containing DNA fragments are generated after enzyme digestion and no single rearranged band is at a level that is detectable. The peripheral blood mononuclear cells of the patient were obtained in 1982 when the patient had increased numbers of T8+, LGL with anemia and neutropenia and in 1984 when the patient had a normalization of the T-cell imbalance with recovery from the neutropenia. In the 1982 mononuclear cell preparation, the T_β constant gene probe hybridized with two *Bam*H1 bands of 14 and 10.5 kb, not seen with germline tissues (Fig 2). This was associated with a diminution in the proportion of DNA hybridizing to the T_β probe that was in the germline pattern. When the radioautograph was analyzed by densitometry, 17% of the DNA hybridizing with the radiolabeled probe was at the 24 kb band with the patient sample as compared with over 96% with the control. The identification of two prominent rearranged bands indicates that there had been effective or aberrant rearrangements of both T_β alleles in a clonal T-cell population. The pattern of T_β gene rearrangements observed with the patient is comparable to that seen in 14 other cases of clonal T-cell leukemias, including the Sezary leukemia,

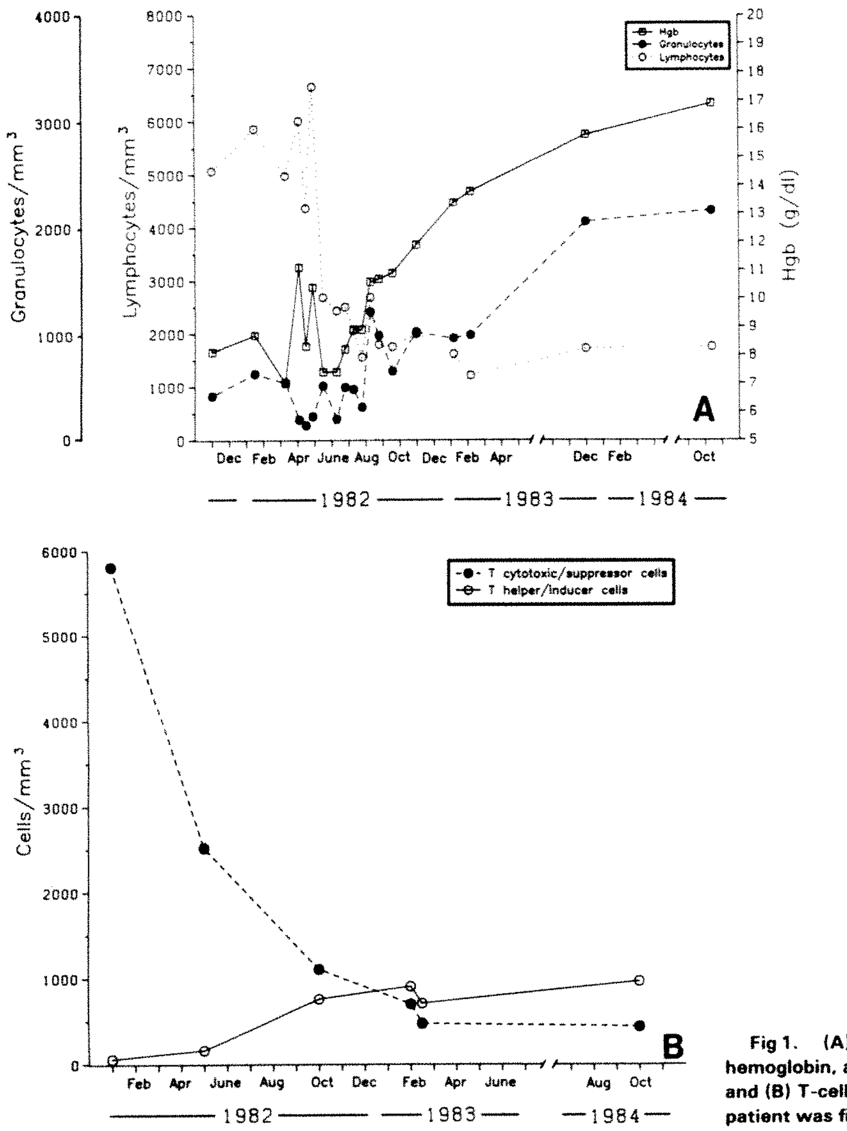

Fig 1. (A) The temporal relationship of peripheral blood hemoglobin, absolute lymphocyte, and granulocyte counts, and (B) T-cell subsets are demonstrated from the time the patient was first diagnosed through October 1984.

adult T-cell leukemia, and acute T-cell precursor lymphoblastic leukemia.[39] The Southern analysis of the cells of the patient obtained during remission in 1984 showed a marked diminution of the intensity of the 14 and 10.5 kb bands relative to the intensity of the 24 kb germline band, consistent with the majority of the circulating T-cells being polyclonal at this time. Residual clonal T-cells remained present in 1984 that could be identified by this molecular approach but could not be identified by analysis of the cell surface phenotype. In summary of the molecular analysis, the patient had a circulating population of clonal T lymphocytes that was the predominant mononuclear cell when he manifested T-cell imbalance and neutropenia and was only a minority population when the patient was in clinical remission.

DISCUSSION

Southern blot analysis to detect T_β gene rearrangements provides a new, more definitive method to determine whether T-cell proliferations are monoclonal. Such clonal rearrangements are universal in mature T-cell lymphomas and leukemias.[31-40] In patients with excess LGL and neutropenia, clonality defined by T_β gene rearrangements has been described by Aisenberg et al in 2 of 3 patients,[36] and seen in 6 of 8 patients studied by the authors (Waldmann TA, Winton EF, unpublished observations).[39] Cytogenetic analysis has also been used to demonstrate clonality of excess LGL in 4 patients with neutropenia, with or without anemia.[43,44]

There has been conflicting opinion as to whether patients with excess T8+, LGL with anemia and/or neutropenia represent a T-cell leukemia or a benign reactive lymphoproliferation. The fact that the patient we describe had a hematologically normal twin suggests that the excess T8+, LGL resulted from an acquired monoclonal proliferation. While monoclonality is most consistent with neoplasia, it does not exclude the possibility of a reactive process.

Despite clonal origin of LGL in some neutropenia patients, we are uncertain that this should be considered a T-cell malignancy in view of the generally benign, nonprogressive course. The spontaneous regression of the monoclonal LGL seen in this patient is a demonstration of the benign potential

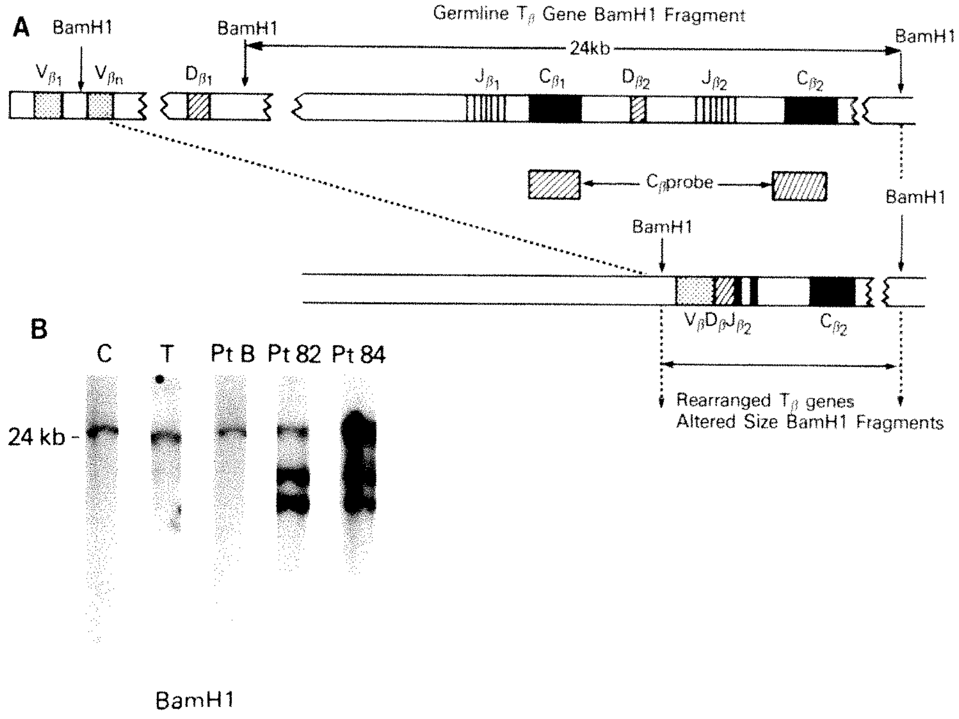

Fig 2. (A) T_β constant region probes utilized to detect germline and rearranged T_β genes. In their germline form the gene segments encoding the two T_β constant genes were present on a single 24 kb *BamH1* fragment. (B) Using Southern analysis the constant regions of the T-cell receptor chain gene were shown to be present on a 24 kb *BamH1* fragment in a white blood cell preparation of a normal individual (lane C) that reflects the arrangement in germline tissues. An identical pattern was achieved in *BamH1* digestion of the DNA from an EBV-induced B-cell line of the patient (PtB). In DNA from the patient's mononuclear cells (predominantly T8 cells) studied in 1982 (Pt-82), there were two new bands observed (indicated by the arrows) of 14 and 10.5 kb. These bands were not seen with the patient's B cells, and were associated with a relative diminution in the intensity of the germline 24 kb band. In 1984 (Pt-84), when the patient was in clinical remission, the 14.0 and 10.5 kb bands reflecting the clonal rearrangements were still evident, but were diminished in intensity relative to the 24 kb band when compared with the 1982 pattern.

of this T-cell proliferation. This observation is not unique, and has been seen by the authors in one similar case (Winton, EF, unpublished observation) and in 3 additional patients described by others.[44,45]

We have referred to the regression of the T-cell clone in our patient as spontaneous because we cannot identify a definite cause. He did not receive chemotherapy or immunosuppressive medications at any time. Theoretically, an immunoregulatory imbalance could have been perturbed by several things that occurred within three to six months of the remission, including an episode of septicemia, multiple packed red blood cell transfusions, and leukapheresis. Another patient with T8+, LGL proliferation and neutropenia has had multiple, serious bacterial infections and undergone leukapheresis to collect cells for research with no subsequent change in the disease status (Winton EF, unpublished observations).

The demonstration of monoclonality of T8+, LGL in this and similar patients suggests that the T-cell proliferation could be the primary event, and the anemia and/or neutropenia secondary. The temporal correlations of clonal regression and reversal of anemia and neutropenia in the patient reported also implicate the LGL in the pathogenesis of the hematocytopenias. Initial attempts to demonstrate T8+, LGL hematopoietic cell interactions in vitro have not been successful with several groups reporting that removal of T cells from the marrow of similar patients did not enhance in vitro granulocyte-macrophage colony growth.[4,9,19] Bagby and coworkers, on the other hand, have reported enhanced granulocyte-macrophage colony growth in certain patients with granulocytopenia (and aplastic anemia) following T-cell removal, yet it is unreported whether these patients had excess T8+, LGL.[46] Several investigators have described patients with anemia with excess LGL and demonstrated inhibition of in vitro erythropoiesis by the LGL.[5,10,19,47] Of further interest, Lipton and coworkers have shown this interaction to be MHC restricted.[47]

Neutrophil antibodies have been demonstrated in some cases of neutropenia with LGL excess.[2,9,43] In addition, there is a single report of neutrophil directed, antibody-dependent cellular cytotoxicity mediated by T_γ cells in a neutropenic patient.[48] Neutrophil directed antibody-dependent cellular cytotoxicity has been noted in 2 other patients with neutropenia, but the interacting lymphoid populations were not characterized.[49]

While most of the above observations are consistent with the pathogenic role for T8+, LGL in these neutropenic patients, further in vitro investigations are needed to better define possible direct, antibody or lymphokine-mediated interactions of LGL and hematopoietic cells. Also, as more of these cases are described, clinical and cellular heterogeneity is becoming apparent in patients with cytopenias and excess LGL. LGL from cases for future in vitro function studies should be characterized as fully as possible regarding cell lineage and monoclonality.

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REFERENCES

1. McKenna RW, Parkin J, Kersey JH, Gajl-Peczalska, Peterson L, Brunning RD: Chronic lymphoproliferative disorder with unusual clinical, morphologic, ultrastructural and membrane surface marker characteristics. Amer J Med 62:588, 1977

2. Bom-Van Noorloos AP, Pegals HG, van Oers R, et al: Proliferation of T gamma cells with killer-cell activity in two patients with neutropenia and recurrent infections. N Engl J Med 302:933, 1980

3. Pandolfi F, Strong DM, Slease RB, Smith ML, Ortaldo JR, Herberman RB: Characterization of a suppressor T-cell chronic lymphocytic leukemia with ADCC but not NK activity. Blood 56:653, 1980

4. Linch DC, Cawley JC, Worman CP, et al: Abnormalities of T-cell subsets in patients with neutropenia and an excess of lymphocytes in the bone marrow. Br J Haematol 48:137, 1981

5. Nagasawa T, Abe T, Nakagawa T: Pure red cell aplasia and hypogammaglobulinemia associated with Tr-cell chronic lymphocytic leukemia. Blood 57:1025, 1981

6. Callard RE, Smith CM, Worman C, Linch D, Cawley JC, Beverley PCL: Unusual phenotype and function of an expanded subpopulation of T cells in patients with hematopoietic disorders. Clin Exp Immunol 43:497, 1981

7. Aisenberg AC, Wilkes BM, Harris NL, Ault KA, Carey RW: Chronic T-cell lymphocytosis with neutropenia: Report of a case studied with monoclonal antibody. Blood 58:818, 1981

8. Hooks JJ, Haynes BF, Detrick-Hooks B, Diehl LF, Gerrard TL, Fauci AS: Gamma (immune) interferon production of leukocytes from a patient with a T_γ cell proliferative disease. Blood 59:198, 1982

9. Starkebaum G, Martin PJ, Singer JW, et al: Chronic lymphocytosis with neutropenia: Evidence for a novel, abnormal T-cell population associated with antibody-mediated neutrophil destruction. Clin Immunol Immunopathol 27:110, 1983

10. Hocking WG, Singh R, Schroff R, Golde DW: Cell mediated inhibition of erythropoiesis and megaloblastic anemia in T-cell chronic lymphocytic leukemia. Cancer 51:631, 1983

11. Chan WC, Winton EF, Check IJ: T-cell imbalance in neutropenia of uncertain etiology. Amer J Clin Pathol 81:54, 1983

12. Chan WC, Schick CC, Brynes RK, Kateley J, Winton EF: A morphologic and immunologic study of the large granular lymphocyte in neutropenia with T lymphocytosis. Blood 63:1133, 1984

13. Brisbane JU, Berman LD, Osband ME, Neiman RS: T8 chronic lymphocytic leukemia: A distinctive disorder related to T8 lymphocytosis. Amer J Clin Pathol 80:391, 1983

14. Phyliky RL, Li CY, Yam LT: T-cell chronic lymphocytic leukemia with morphologic and immunologic characteristics of cytotoxic/suppressor phenotype. Mayo Clin Proc 58:709, 1983

15. Palutke M, Eisenberg L, Kaplan J, et al: Natural killer and suppressor T-cell chronic lymphocytic leukemia. Blood 62:627, 1983

16. Itoh K, Tsuchikawa K, Awataguchi T, Shiiba K, Kumagai K: A case of chronic lymphocytic leukemia with properties characteristic of natural killer cells. Blood 61:940, 1983

17. Herrmann F, Sieber G, Kominschke B, Schreckenberg A, Ludwig W-D, Ruhl H: Expanded T_γ cell populations with the morphology of large granular lymphocytes. I. Immunologic clinical and morphological characterization. Clin Exp Immunol 52:641, 1983

18. Bakri K, Ezdinli EZ, Wasser LP, et al: T-suppressor cell chronic lymphocytic leukemia: Phenotypic characterization by monoclonal antibodies. Cancer 54:284, 1984

19. Newland, AC, Catovsky D, Linch D, et al: Chronic T cell lymphocytosis: A review of 21 cases. Br J Haematol 58:433, 1984

20. Reynolds CW, Foon KA: Review. T_γ—Lymphoproliferative disease and related disorders in humans and experimental animals: A review of the clinical, cellular, and functional characteristics. Blood 64:1146, 1984

21. Korsmeyer SJ, Arnold A, Bakhshi A, et al: Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T-cell and B-cell precursor origins. J Clin Invest 71:301, 1983

22. Arnold A, Cossman J, Bakhshi A, Jaffe ES, Waldmann TA, Korsmeyer SJ: Immunoglobulin gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J Med 309:1593, 1983

23. Allison JP, McIntyre BW, Block D: Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. J Immunol 129:2293, 1982

24. Haskins K, Kubo R, White J, Pigeon M, Kappler J, Marrack P: The major histocompatibility complex restricted antigen receptor on T cells. Isolation with a monoclonal antibody. J Exp Med 157:1149, 1983

25. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL: Clonotypic structures involved in antigen-specific human T-cell function. J Exp Med 157:705, 1983

26. Hedrick SM, Cohen DI, Nielsen EA, Davis MM: Isolation of cDNA clones encoding T-cell specific membrane-associated proteins. Nature 308:149, 1984

27. Hedrick SM, Nielsen EA, Kavaler J, Cohen DI, Davis MM: Sequence relationships between putative T-cell receptor, polypeptides and immunoglobulins. Nature 308:153, 1984

28. Yanagi Y, Yoshikai Y, Leggett R, Clark SP, Aleksander I, Mak TW: A human T-cell specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature 308:145, 1984

29. Chien YH, Becker DM, Lindsten T, Okamuras M, Cohen DI, Davis MM: A third type of murine T-cell receptor gene. Nature 312:31, 1984

30. Saito H, Kranz DM, Takagki Y, Hayday AC, Eisen HN, Tonegawa S: A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. Nature 312:36, 1984

31. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T: Somatic rearrangement of T-cell antigen receptor gene in human T-cell malignancies. Proc Natl Acad Sci USA 82:1224, 1985

32. Flug F, Pelicci P-G, Bonetti F, Knowles DM II, Dalla-Favera R: T-cell receptor gene rearrangement as markers of lineage and clonality in T-cell neoplasms. Proc Natl Acad Sci USA 82:3460, 1985

33. Yanagi N, Caccia N, Kronenberg M, Chin B, Roder J, Rohel D, Kiyohara T, Lauzon R, Toyonaga B, Rosenthal K, Dennert G, Acha-Orbea H, Hengartner H, Hood L, Mak TW: Gene rearrangement in cells with natural killer activity and expression of the beta-chain of the T-cell antigen receptor. Nature 314:631, 1985

34. Ritz J, Campen TJ, Schmidt RE, Royer HD, Hercend T, Hussey RE, Reinherz EL: Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. Science 228:1540, 1985

35. Toyonaga B, Yanagi Y, Suciu-Foca N, Minden M, Mak T: Rearrangements of T-cell receptor gene YT35 in human DNA from thymic leukemia T-cell lines and functional T-cell clones. Nature 311:385, 1984

36. Aisenberg AC, Krontiris TG, Mak TW, Wilkes BM: Rearrangement of the gene for the beta chain of the T-cell receptor in T-cell chronic lymphocytic leukemia and related disorders. N Engl J Med 313:529, 1985

37. Bertness V, Kirsch I, Hollis G, Johnson B, Bunn PA Jr: T-cell receptor gene rearrangements as clinical markers of human T-cell lymphomas. N Engl J Med 313:534, 1985

38. Weiss LM, Hu E, Wood GS, Moulds C, Cleary ML, Warnke R, Sklar J: Clonal rearrangements of T-cell receptor genes in mycosis fungoides and dermatopathic lymphadenopathy. N Engl J Med 313:539, 1985

39. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ: Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 313:776, 1985

40. Tawa A, Hozumi N, Minden M, Mak TW, Gelfand EW: Rearrangement of the T-cell receptor beta-chain gene in non-T-cell, non-B-cell acute lymphoblastic leukemia of childhood. N Engl J Med 313:1033, 1985

41. Abo T, Miller CA, Gartland GL, Balch CM: Differentiation stages of human natural killer cells in lymphoid tissue from fetal to adult life. J Exp Med 157: 273, 1983

42. Hercend T, Griffin JD, Bensussan A, et al: Generation of monoclonal antibodies to a human natural killer clone: Characterization of two natural killer associated antigens, NKH1A and NKH2, expressed on subsets of large granular lymphocytes. J Clin Invest 75:932, 1985

43. Loughran TP Jr, Kadin ME, Starkebaum G, Abkowitz JL, Clark EA, Disteche C, Lum LG, Slichter SJ: Leukemia of large granular lymphocytes: Association with clonal chromosomal abnormalities and autoimmune neutropenia, thrombocytopenia, and hemolytic anemia. Ann Intern Med 102:69, 1985

44. McKenna RW, Arthur DC, Gajl-Peczalska KJ, Flynn P, Brunning RD: Granulated T-cell lymphocytosis with neutropenia: Malignant or benign chronic lymphoproliferative disorder? Blood 66:259, 1985

45. Pandolfi F, Semenzato G, de Rossi G: Chronic lymphocytosis due to the expansion of granular lymphocytes. Brit J Haematol 60:771, 1985

46. Bagby GC Jr, Lawrence JH, Neerhout RC: T-lymphocyte-mediated granulopoietic failure. In vitro identification of prednisone-responsive patients. N Engl J Med 309:1073, 1983

47. Lipton JM, Nadler LM, Canellos GP, Kudisch M, Reiss CS, Nathan DG: Evidence for genetic restriction in the suppression of erythropoiesis by a unique subset of T lymphocytes in man. J Clin Invest 72:694, 1984

48. Pross HF, Pater DI, Giles A, Gallinger LA, Rubin P, Corbett WEN, Galbraith P, Baines MG: Studies of human natural killer cells. III. Neutropenia associated with unusual characteristics of antibody-dependent and natural killer cell-mediated cytotoxicity. J Clin Immunol 2:126, 1982

49. Logue GL, Kurlander R, Pepe P, Davis W, Silberman H: Antibody-dependent lymphocyte-mediated granulocyte cytotoxicity in man. Blood 51:97, 1978

Establishment of Eosinophilic Sublines From Human Promyelocytic Leukemia (HL-60) Cells: Demonstration of Multipotentiality and Single-Lineage Commitment of HL-60 Stem Cells

By Masao Tomonaga, Judith C. Gasson, Shirley G. Quan, and David W. Golde

Recent observations indicating that the HL-60 human acute promyelocytic leukemia cell line contains a minor eosinophil population in addition to neutrophil and mononuclear phagocyte progenitors suggest the multipotentiality of HL-60 stem cells. In order to clarify multilineage differentiation and commitment to single-lineage progenitors we analyzed HL-60 colonies formed in methylcellulose. In an HL-60 parent line with a relatively high eosinophil content (5.5%), 36% of the spontaneous colonies consisted partly or wholly of eosinophilic cells. After two rounds of subcloning in methylcellulose, two eosinophilic sublines and two neutrophilic sublines were established. These lines have been in continuous liquid culture for more than four months, and they show stable single-lineage differentiation. Purified biosynthetic GM-CSF, which stimulates normal CFU-GM and CFU-EO, induced monocytic differentiation but no eosinophilic differentiation in the neutrophilic sublines and no neutrophilic or monocytic differentiation in the eosinophilic sublines. These observations indicate that HL-60 stem cells are multipotent and capable of spontaneous commitment to single-lineage progenitors. The eosinophilic HL-60 sublines should facilitate studies on the production and function of human eosinophils and the single-lineage sublines will allow further analysis of leukemic cell differentiation and stem cell commitment.

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THE HL-60 cell line is a human myeloid cell line derived from a patient with acute promyelocytic leukemia.[1] The cytologic characteristics of HL-60 cells and the original fresh leukemic cells are those of neutrophilic promyelocytes. They exhibit strong myeloperoxidase and specific esterase activities.[2] HL-60 cells form spontaneous colonies in semisolid media and respond to human granulocyte-macrophage colony-stimulating factor (GM-CSF).[3-5]

Many studies have shown that HL-60 cells can be induced by a variety of agents to differentiate toward either more mature neutrophil-like cells or monocyte-macrophage-like cells.[6-10] Based on these results, it has been postulated that the progenitor cells (stem cells) of HL-60 show bilineage differentiation similar to normal granulocyte-macrophage progenitor cells (CFU-GM).[11,12] Recently, however, eosinophil-like cells have been noted as a minor population of HL-60 cells.[13-16] It was demonstrated that they have many of the characteristics of normal eosinophils.[15] Thus, HL-60 seems to be capable of multilineage differentiation.

In our previous study on the biological activity of human biosynthetic (recombinant) GM-CSF on normal bone marrow cells and myeloid leukemia cell lines, the finding of eosinophilic differentiation in HL-60 was confirmed.[5] We observed the appearance of cells with distinct eosinophilic granules in liquid and methylcellulose culture without GM-CSF, and also a consistent increase of such cells in the presence of GM-CSF. Therefore, we attempted to select highly eosinophilic subclones from HL-60 in order to clarify whether a process of commitment to single lineage occurs in the multipotent HL-60 stem cell compartment. After two successive subclonings, we obtained highly eosinophilic sublines that have been maintained for more than four months in the absence of GM-CSF. These sublines show most of the cytological features of normal eosinophils. Along with the establishment of eosinophilic sublines, we also obtained highly neutrophilic sublines that lack eosinophilic differentiation. The establishment of such single-lineage sublines from parent HL-60 cells capable of trilineage differentiation indicates the multipotentiality of HL-60 stem cells and their spontaneous commitment to single-lineage differentiation.

MATERIALS AND METHODS

Cells. Liquid culture experiments were performed using Iscove's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were passaged at weekly intervals when cell concentrations reached 1.5×10^6 cells/mL. In these studies, we primarily used HL-60 passage 50. Frozen stocks of early passage HL-60 (P-25 and P-29) were used for some experiments.

Clonal culture in methylcellulose. HL-60 cells were harvested from liquid culture on the second day after refeeding when they were in exponential growth. Cells (3×10^2) were plated in Falcon microtiter wells (Becton-Dickinson, Oxnard, Calif) containing 0.1 mL Iscove's medium with 0.92% methylcellulose (Dow Chemical, Midland, Mich), 10% fetal bovine serum (selected lot) and antibiotics, and incubated at 37 °C in a fully humidified atmosphere of 7% CO_2 in air. On day 8, cell aggregates containing 40 cells or more were enumerated as colonies.

Subcloning of HL-60 cells. Random aspiration of individual colonies growing in methylcellulose was undertaken on day 8 of culture using a sterile, finely drawn-out microcapillary. Each colony was transferred into 0.2 mL of complete Iscove's medium in a Falcon microtiter well and maintained by refeeding one-half the volume of the well every four days until the cell number reached 1×10^5 (usually for two weeks). Each subclone was then transferred to a one mL culture in a larger well to allow continued growth to a cell density of 1×10^6 (for a further two weeks) and used for cytochemical analysis and secondary subcloning experiments. Four subclones which exhibited single-lineage differentiation were maintained in liquid culture over three months to confirm the stability of their phenotype.

Granulocyte-macrophage colony-stimulating factor (GM-CSF). Purified biosynthetic human GM-CSF was obtained from

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COS monkey kidney cells transfected with the GM-CSF cDNA clone in the expression vector p91023 (B).[17] Serum-free conditioned medium was collected for three days. The GM-CSF was purified from the conditioned medium by a modification of the previously designated method,[18] omitting lectin affinity chromatography and using gel filtration and reverse phase high performance liquid chromatography. The purified GM-CSF appeared as a single protein band of 22,000 molecular weight by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis. The specific activity of the GM-CSF on normal human bone marrow cells was 2×10^8 units per mg: half maximal activity on bone marrow cells was seen at 10^{-11} mol/L.[5] GM-CSF, diluted with phosphate-buffered saline containing 0.01% BSA, was added to methylcellulose culture or liquid culture to give final concentrations from 31.2 pmol/L to 1.0 nmol/L.

Cytological analysis. The cellular composition of HL-60 parent lines and their subclones was determined by staining the cells using the method of Li et al for double esterases[5,19] and with Biebrich scarlet (B-s).[5,20] Cells from liquid cultures were washed once with Iscove's medium and resuspended in the medium. Aliquots (10-μL) were placed on a poly-L lysine-coated microscope slide and the cells allowed to settle for 10 minutes.[5] Excess medium was removed with absorbent paper. The slides were rapidly air-dried and fixed for 30 seconds at 7 °C with phosphate-buffered formalin-acetone. Individual HL-60 colonies were aspirated and transferred into a 10-μL droplet of Iscove's medium placed on poly-L lysine-coated slides and fixed.

Only cells with moderate (+ +) to strong (+ + +) activity of naphthol-ASD chloracetate esterase (Es-chl) were regarded as neutrophilic granulocytes because eosinophilic HL-60 cells also showed weak (±, +) activities. Cells positive for alpha-naphthyl butyrate esterase (Es-b) were classified as monocytes and cells with distinct granules positive for B-s stain were classified as eosinophils. The eosinophilic nature of the cells with B-s–positive granules was further confirmed by demonstrating staining wtih Luxol fast blue[21] and cyanide-resistant myeloperoxidase activity.[22,23] Myeloperoxidase was detected using diaminobenzidine as substrate. Cyanide-resistant myeloperoxidase was demonstrated by adding 4.9 mg NaCN to 10 mL of reaction medium.

Differentiation-induction by chemical inducers and GM-CSF. Based on previous reports on the induction of differentiation in HL-60,[6-10] three chemical compounds, dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), and tetradecanoyl phorbol acetate (TPA), were used to examine their effects on the differentiation of single-lineage HL-60 sublines. Purified biosynthetic GM-CSF was employed as a physiologic inducer.[5] One hundred μL (10%) of dilutions of inducer was added to duplicate 1-mL liquid cultures containing 1×10^5 cells in complete Iscove's medium. DMSO was added to give final concentrations of 1.25%, 0.625%, and 0.312%; RA, 1×10^{-6} mol/L, 1×10^{-7} mol/L, and 1×10^{-8} mol/L; TPA, 1×10^{-7} mol/L, 5×10^{-8} mol/L, and 1×10^{-8} mol/L; GM-CSF, 1 nmol/L, 250 pmol/L, and 31.2 pmol/L, respectively. On day 7, cells were enumerated and poly-L lysine smears prepared and stained for cytochemical analysis. Alkaline phosphatase staining (Kit No. 85, Sigma, St. Louis) and nitroblue tetrazolium (NBT) dye reduction (Sigma Kit No. 840) were also used to assess cell differentiation.

RESULTS

Table 1. Eosinophilic Differentiation of HL-60 Parent Lines in Liquid Culture

Passage Number	Concentration of GM-CSF (pmol/L)	Cell Number per Well ($\times 10^3$)	Cell Composition Es-chl$^+$ (%)	Es-b$^+$ (%)	B-s$^+$ (%)
P-29					
	250	58.7 ± 5.7	34.0	9.0	4.5
	125	55.7 ± 7.5	54.5	6.0	3.5
	62.5	72.5 ± 17.5	54.0	5.5	2.5
	31.25	55.0 ± 10.3	56.0	2.5	1.0
	15.625	50.7 ± 12.6	58.0	0.5	2.0
	PBS	23.7 ± 14.2	69.5	0.0	1.5
P-50					
	250	105.2 ± 7.2	42.0	13.0	13.0
	125	112.5 ± 9.6	52.5	11.0	11.5
	62.5	146.3 ± 7.4	56.5	12.5	10.0
	31.25	132.6 ± 5.4	55.0	7.0	9.0
	15.625	128.2 ± 11.2	59.5	2.0	8.5
	PBS	78.2 ± 8.4	62.0	1.5	5.5

Liquid cultures were performed in triplicate in microtiter plates containing 0.1 mL. Each well initially contained 3×10^3 cells. After seven days of incubation, cells were enumerated by trypan blue dye exclusion, and smears were prepared from two wells. Percentages of Es-chl–positive cells (neutrophilic cells) and Es-b–positive cells (monocytic cells) were calculated by counting 200 cells on double esterase stained smears, and B-s–positive cells (eosinophilic cells) by counting 200 cells of B-s stained smear. Es-chl, naphthol ASD-chloracetate esterase; Es-b, alpha-naphthyl butyrate esterase; B-s, Biebrich scarlet; PBS, phosphate-buffered saline.

Eosinophilic differentiation in HL-60 parent lines. To examine spontaneous and GM-CSF–induced eosinophilic differentiation of HL-60 cells, two different passages (P-29 and P-50) were cytochemically analyzed after seven days' culture. As shown in Table 1, control cultures (with phosphate-buffered saline) of the two passages exhibited consistent eosinophilic differentiation; B-s–positive eosinophilic cells were 5.5% in P-50 and 1.5% in P-29. Most of the eosinophilic cells were in the promyelocyte stage and had distinct granules positive for B-s. Band and segmented forms with coarse B-s–positive granules were occasionally observed, especially in P-50. In response to increasing concentrations of GM-CSF, both passages showed a proportionate increase in the percentage of eosinophils. In addition, the eosinophils present showed a greater degree of maturation. Es-b–positive monocytic elements appeared for the first time after GM-CSF stimulation, and increased in percentage proportionately to GM-CSF concentrations.

Clonal analysis of eosinophilic differentiation in HL-60 parent line. Individual colonies formed by the two passages of the HL-60 parent line in methylcellulose culture with or without GM-CSF were aspirated and stained with B-s. As shown in Table 2, the total number of colonies increased proportionately with GM-CSF concentration. In the presence of 250 pM GM-CSF, P-29 showed 5% eosinophil-containing colonies in which eosinophils comprised less than 20% of the cells in each individual colony. Without GM-CSF, no eosinophilic colonies were found among 40 colonies analyzed. In contrast, P-50 exhibited a higher frequency of eosinophilic colony in the control culture, and GM-CSF increased the incidence further. The eosinophil content in single colonies varied widely from 3% to 100%.

Primary subcloning of HL-60 parent line. Primary subcloning of individual colonies formed by P-50 was undertaken. Fifty colonies were aspirated on day 8 from cultures grown with and without GM-CSF. Colony size varied from 40 to 400 cells. Each colony was transferred into liquid culture without GM-CSF and maintained until the cell

Table 2. Eosinophilic Differentiation in Parent HL-60 Colonies Growing in Methylcellulose Culture

Passage Number	Concentration of GM-CSF (pmol/L)	Colony Number per Well (mean ± SE)	Total No. of Eosinophil-Containing Colonies (%)	Percentage of Eosinophils in Individual Colonies				
				3–20	21–50	51–80	81–99	100
P-29								
	250	74 ± 3.7	2/40 (5.0)	2	0	0	0	0
	31.2	34.3 ± 4.9	0/20 (0.0)	—	—	—	—	—
	PBS	18.3 ± 5.7	0/40 (0.0)	—	—	—	—	—
P-50								
	250	75 ± 6.0	34/77 (44.0)	11	5	10	6	2
	31.2	40 ± 3.2	18/45 (40.0)	9	4	2	2	1
	PBS	32.5 ± 2.3	17/47 (36.2)	6	3	3	4	1

Triplicate methylcellulose cultures were undertaken. After seven days, colonies were enumerated and slide smears were made and stained with Biebrich scarlet. Colonies with more than three eosinophilic cells showing distinct positive granules were regarded as eosinophil-containing. Eosinophil-content (%) was determined by counting all colony-composing cells for Biebrich scarlet staining. PBS, phosphate-buffered saline.

number reached 1 × 10^6. Eight subclones originally derived from the GM-CSF–containing culture, and six subclones derived from the culture without GM-CSF were analyzed further. These 14 subclones were studied cytochemically for their cellular composition. As shown in Table 3, 12 subclones showed bilineage differentiation as evidenced by the presence of both Es-chl–positive neutrophilic cells and B-s–positive eosinophilic cells. No subclones exhibited pure eosinophilic differentiation. Two subclones, 8 and 14, however, showed highly neutrophilic differentiation. The bilineage subclones showed a wide range in eosinophil content, No. 3 showing the highest percentage of 78%, and No. 6, the lowest of 3%.

Secondary subcloning of highly eosinophilic subclone No. 3. In an effort to develop pure eosinophil lines, secondary subcloning of clone No. 3 was undertaken using the same methods as in primary subcloning. Subclone No. 3 showed a similar cloning efficiency to the parent, P-50. Twenty-five colonies were randomly aspirated from the culture without GM-CSF, of which 23 subclones reached 1 × 10^6 cells after three weeks. As shown in Table 4, cytochemical analysis of these secondary subclones revealed that most of them were highly eosinophilic. Thirteen sub-

Table 3. Primary Subcloning from Parent HL-60 Passage 50

Subclone Number	GM-CSF in Methylcellulose Culture	Cell Composition		
		Es-chl$^+$ (%)	Es-b$^+$ (%)	B-s$^+$ (%)
1	+	41	0	56
2	+	51	0	34
3	+	29	0	78
4	+	40	0	47
5	+	68	0	6
6	+	59	0	3
7	+	29	0	12
8	+	79	0	0
9	–	42	0	6
10	–	43	0	16
11	–	53	0	11
12	–	57	0	12
13	–	48	0	30
14	–	62	0	0

Primary subcloning was undertaken by aspirating individual colonies grown in the methylcellulose culture with or without GM-CSF (250 pmol/L). Each clone was maintained in liquid culture until reaching 1 × 10^6 cells. Poly-L lysine-slide smears were made and stained using the double esterase method and Biebrich scarlet. Es-chl-positive (neutrophilic) cells and Es-b-positive (monocytic) cells were calculated by counting 200 cells on a double esterase stained smear and B-s-positive (eosinophilic) cells by counting 200 cells on a Biebrich scarlet stained smear. Es-chl, naphthol-ASD chloroacetate esterase; Es-b, alpha-naphthyl butyrate esterase; B-s, Biebrich scarlet.

Table 4. Secondary Subcloning from Primary Subclone No. 3

Secondary Subclone Number	Cell Composition			
	Es-chl$^+$ (%)	B-s$^+$ (%)	MPO$^+$ (%)	CN-MPO$^+$ (%)
(1)	0	100	100	100
(2)	0	100	100	100
(3)	0	100	100	100
(4)	0	100	100	100
(5)	0	100	100	100
(6)	0	100	100	99
(7)	0	100	100	99
(8)	0	100	100	98
(9)	0	100	100	98
(10)	0	100	100	98
(11)	0	100	100	97
(12)	0	100	100	97
(13)	0	100	100	97
(14)	0	99	100	95
(15)	0	99	100	95
(16)	0	99	100	95
(17)	0	99	100	95
(18)	0	99	100	94
(19)	0	99	100	93
(20)	0	99	100	93
(21)	0	97	100	93
(22)	0	95	100	92
(23)	12	86	100	85

Secondary subclones of highly eosinophilic primary subclone No. 3 were analyzed by aspirating individual colonies grown in methylcellulose culture without GM-CSF. Each clone was maintained in liquid culture until reaching 1 × 10^6 cells and smears were stained with double esterase, Biebrich scarlet (B-s), myeloperoxidase (MPO), and cyanide-resistant myeloperoxidase (CN-MPO). On each cytochemical smear, one hundred cells were counted for a positive reaction. Only cells showing moderate (+ +) to strong (+ + +) diffuse cytoplasmic stain for naphthol-ASD chloroacetate esterase (Es-chl) were regarded as neutrophilic, because most of the eosinophilic HL-60 cells showed weak (+) positivity for this enzyme.

clones showed 100% of the cells positive for B-s stain seven days after refeeding. The remaining 10 subclones were 86 to 99% positive for B-s. Clone No. 3 (23) was the only one with a lower (86%) eosinophil content and a minor population (12%) of Es-chl–positive neutrophilic cells. This implied that No. 3 (23) was the only subclone which manifested bilineage differentiation like its parent P-50. Myeloperoxidase staining with or without CN was also used to analyze these secondary subclones. All of them were 100% positive for myeloperoxidase. Most of them showed very high percentages of cells (92-100%) positive for cyanide-resistant myeloperoxidase, indicating that they were highly eosinophilic clones. No. 3 (23), however, exhibited a lower percentage (85%) of cyanide-resistant myeloperoxidase, suggesting that cyanide-sensitive neutrophilic cells were present, consistent with the identification of a distinctively Es-chl–positive neutrophil population. From these highly eosinophilic subclones, five subclones (Nos. 1–5), which were 100% positive for both B-s– and cyanide-resistant myeloperoxidase, were selected as candidates for pure eosinophilic HL-60 sublines and maintained in liquid culture.

Subcloning of highly neutrophilic primary subclones Nos. 8 and 14. Primary subclones Nos. 8 and 14 were also subcloned in order to develop pure neutrophilic colonies of mixed colonies containing eosinophils. No. 14 had about the same plating efficiency as the parent P-50, and No. 8 formed colonies half as well. Twenty-five colonies were characterized from each; all subclones showed only neutrophilic differentiation. Subclones 8 and 14 were regarded as pure neutrophilic sublines of HL-60 and were maintained in liquid culture.

Cytologic analysis of single-lineage HL-60 sublines. Thus, we obtained five eosinophilic and two neutrophilic subclones, all of which showed good growth in liquid culture. After three months in liquid culture, cytochemic analysis was performed on two representatives (Nos. 3^4 and 3^5 of the five eosinophilic sublines and the two neutrophilic sublines (Nos. 8 and 14). As shown in Table 5, the eosinophilic sublines again exhibited highly eosinophilic differentiation in control culture (data shown only for No. 3^5). Both were almost 100% positive for B-s (Fig 1A), myeloperoxidase (Fig 1B), and cyanide-resistant myeloperoxidase (Fig 1C), and lacked Es-chl–positive neutrophilic cells. Also, electron microscopy revealed typical eosinophil granules in the cells of the eosinophilic sublines.

Nos. 8 and 14 were again negative for B-s stain (data shown only for No. 14) (Fig 2A). They were highly positive for Es-chl and myeloperoxidase activity (Fig 2B). The latter activity was almost completely inhibited by the addition of cyanide (Fig 2C).

Growth patterns of single-lineage HL-60 sublines in liquid culture and methylcellulose culture. The growth patterns in liquid culture of the HL-60 parent line, P-25, and the sublines were compared. P-25 and No. 3^4 proliferated at the highest rates, reaching cell densities of approximately 2.5×10^6 cells/mL on day 8 and day 6, respectively. No. 3^5 and No. 14 grew at moderate rates and reached a plateau on day 8. No. 12 showed a final cell density of approximately half that of P-25 and No. $3.^4$

Colony-forming capacity in methylcellulose culture in the absence or presence of GM-CSF was also investigated. All sublines formed colonies of considerable size (50 to 200 cells

Table 5A. Differentiation-Induction in HL-60 Parent Line and Single-Lineage Sublines by Chemical Inducers and Purified Human Recombinant GM-CSF: Eosinophilic HL-60 Subline No. 3^5

Inducer Concentration	Cell No. Per Well ($\times 10^6$)	Cytochemic Analysis (%)						Nuclear Maturation (%)	
		B-s$^+$	Es-chl$^+$	Es-b$^+$	MPO$^+$	CN-MPO$^+$	NBT$^+$	Band +Seg	Macrophage
1. Control with PBS									
	1.91	99.5	0	0	100.0	100.0	0	5.0	0
2. Dimethyl sulfoxide									
0.312%	1.62	77.0	2.5	0	99.5	72.5	1.5	8.0	0
0.625%	1.30	49.5	0	0	81.0	54.5	11.5	11.0	0
1.25%	1.30	30.0	0	0	58.0	39.5	48.5	22.0	0
3. Retinoic acid									
10^{-8} mol/L	2.60	80.0	0	0	97.5	83.5	7.5	8.0	0
10^{-7} mol/L	1.15	73.5	0	0	80.5	56.5	17.5	11.5	0
10^{-6} mol/L	0.46	67.0	0	0	48.5	37.5	24.5	27.5	0
4. Tetradecanoyl phorbol acetate									
10^{-8} mol/L	0.20	54.0	0	3.5	5.0	3.5	1.5	0	39.0
5×10^{-8} mol/L	0.10	32.5	0	4.0	7.5	2.5	1.5	0	53.0
10^{-7} mol/L	0.02	22.0	0	3.0	4.5	1.5	1.5	0	80.0
5. GM-CSF									
31.2 pmol/L	2.32	98.5	0	0	100.0	100.0	0	16.0	0
250 pmol/L	2.82	98.0	0	0	99.5	100.0	0.5	19.5	0
1 nmol/L	2.54	98.5	0.5	0	100.0	98.0	1.0	25.0	0

Table 5B. Differentiation-Induction in HL-60 Parent Line and Single-Lineage Sublines by Chemical Inducers and Purified Human Recombinant GM-CSF: Neutrophilic HL-60 Subline No. 14

Inducer Concentration	Cell No. Per Well (× 10^6)	Cytochemic Analysis (%)						Nuclear Maturation (%)	
		B-s$^+$	Es-chl$^+$	Es-b$^+$	MPO$^+$	CN-MPO$^+$	NBT$^+$	Band + Seg	Macrophage
1. Control with PBS	1.28	0	51.0	1.0	100.0	0	6.5	6.0	0
2. Dimethyl sulfoxide									
0.312%	1.94	0.2	48.5	0.5	95.5	0.2	12.5	14.0	0
0.625%	2.41	0	43.0	0.5	81.5	0	22.5	29.0	0
1.25%	0.30	0	39.5	1.5	39.5	0	31.5	41.5	0
3. Retinoic acid									
10^{-8}mol/L	1.27	0.2	58.5	0	97.5	0.2	8.0	13.5	0
10^{-7}mol/L	0.87	0	46.0	0	93.0	0.2	12.5	39.5	0
10^{-6}mol/L	0.56	0	40.0	0	73.5	0	17.0	74.5	0
4. Tetradecanoyl phorbol acetate									
10^{-8}mol/L	0.20	20.0	31.5	23.5	35.0	9.5	2.5	0	15.0
5 × 10^{-8}mol/L	0.10	0	11.5	40.0	0.5	0	3.5	0	28.0
10^{-7}mol/L	0.02	0	7.0	49.0	0.5	0	3.5	0	64.0
5. GM-CSF									
31.2 pmol/L	1.97	$<$ 0.2	46.0	7.5	99.0	0	15.0	9.0	0
250 pmol/L	1.86	0	45.0	10.0	99.0	0	25.0	10.5	0
1 nmol/L	2.19	0	45.5	8.5	100.0	0	33.5	12.5	0

Table 5C. Differentiation-Induction in HL-60 Parent Line and Single-Lineage Sublines by Chemical Inducers and Purified Human Recombinant GM-CSF: HL-60 Parent Line Passage-25

Inducer Concentration	Cell No. Per Well (× 10^6)	Cytochemic Analysis (%)						Nuclear Maturation (%)	
		B-s$^+$	Es-chl$^+$	Es-b$^+$	MPO$^+$	CN-MPO$^+$	NBT$^+$	Band + Seg	Macrophage
1. Control with PBS	1.97	5.5	57.0	1.0	100.0	4.0	5.5	3.5	0
2. Dimethyl sulfoxide									
0.312%	2.18	1.0	53.0	2.5	99.0	1.0	6.0	12.5	0
0.625%	2.74	$<$ 0.2	49.0	2.5	95.5	0.2	18.0	35.0	0
1.25%	0.38	$<$ 0.2	14.0	1.0	92.5	0.2	26.0	12.0	0
3. Retinoic acid									
10^{-8}mol/L	1.93	1.0	52.5	1.0	98.5	1.0	4.5	16.0	0
10^{-7}mol/L	1.60	0.2	45.0	2.0	95.0	0.2	10.5	36.0	0
10^{-6}mol/L	0.59	0	26.0	1.5	88.5	0.2	28.5	66.5	0
4. Tetradecanoyl phorbol acetate									
10^{-8}mol/L	3.35	19.5	33.5	9.5	59.0	10.5	6.5	3.5	5.0
5 × 10^{-8}mol/L	0.10	0	0.5	76.5	4.5	0	8.0	2.0	43.5
10^{-7}mol/L	0.07	0	0	86.5	2.0	0	10.5	0	78.0
5. GM-CSF									
31.2 pmol/L	3.30	7.0	54.5	4.0	99.0	6.0	5.0	4.5	0
250 pmol/L	3.01	12.0	54.5	5.5	99.5	8.0	7.5	4.5	0
1 nmol/L	3.30	13.0	53.0	3.5	98.0	9.5	8.5	8.5	0

Cell number per well and positive rate for each cytochemic stain are given as the mean duplicate cultures. B-s, Biebrich scarlet stain; Es-chl, naphthol-ASD chloroacetate esterase; Es-b, alpha-naphthyl butyrate esterase; MPO, myeloperoxidase; CN-MPO, cyanide-resistant myeloperoxidase; NBT, nitroblue tetrazolium dye reduction.

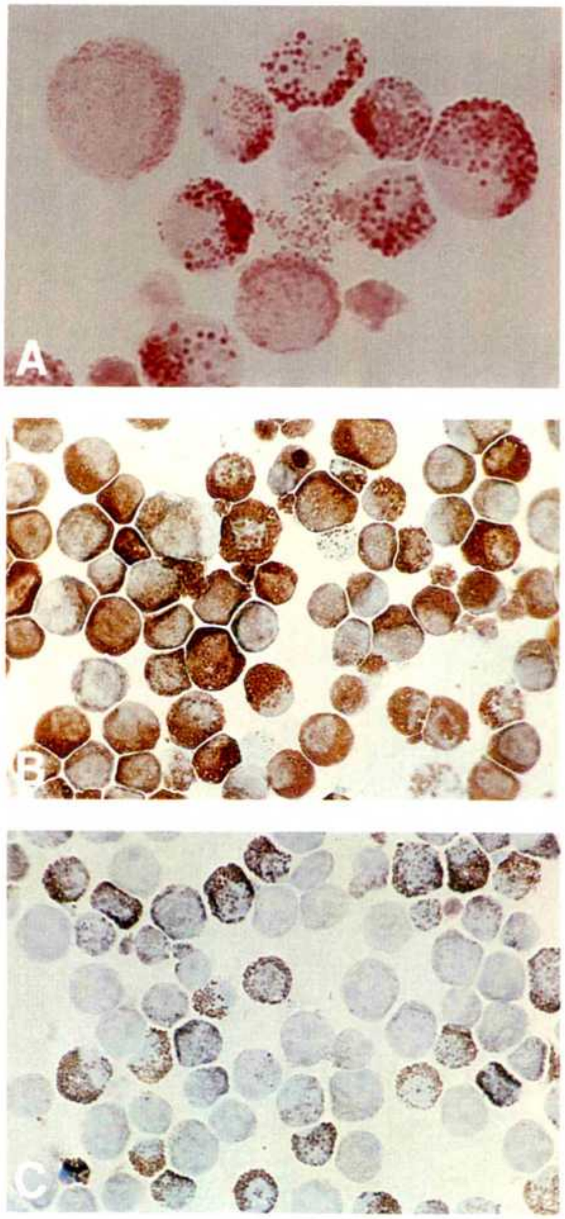

Fig 1. Cytochemic characteristics of the eosinophilic HL-60 subline No. 3.[5] (A) Biebrich-scarlet (B-s) stain (original mag ×500; current mag ×200). Cells harvested seven days after refeeding were all positive for B-s. The size and number of the eosinophilic granules per cell tend to increase with cellular maturation. Not counterstained. (B) Myeloperoxidase stain using diaminobenzidine as substrate (original mag ×250; current mag ×96). Cells are 100% positive and show characteristic coarse positive granules similar to those of normal eosinophils. (C) Cyanide (CN)-resistant myeloperoxidase stain (original mag ×250; current mag ×96). Although the intensity of the reaction is reduced compared with the myeloperoxidase stain without CN, almost all cells remain positive, indicating that they have the eosinophil-type enzyme. Note that the mature forms are especially resistant to CN treatment.

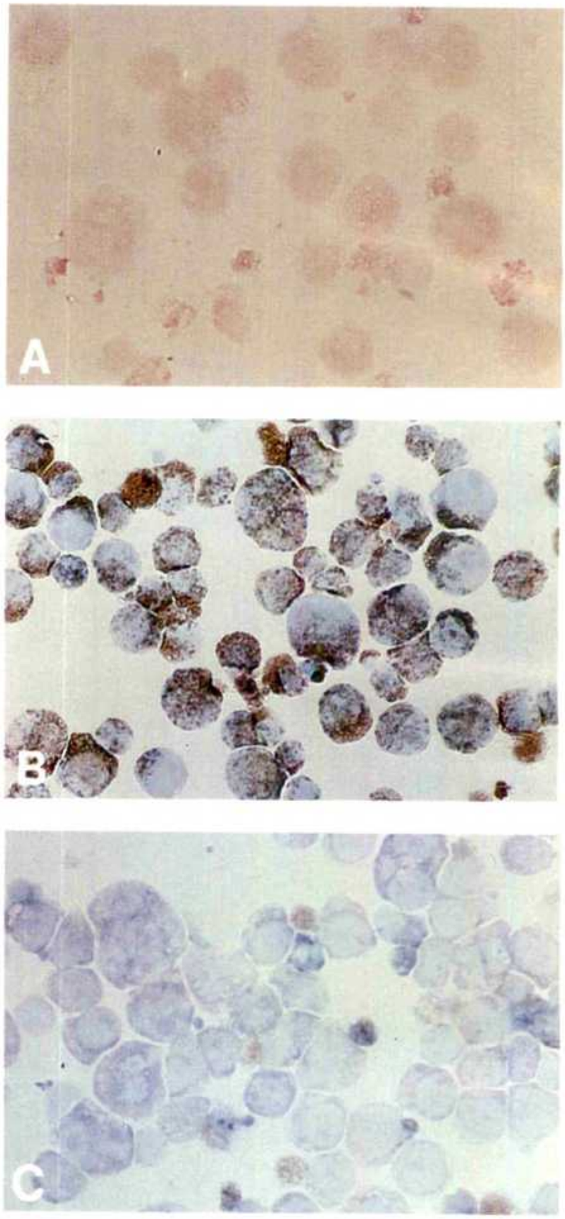

Fig 2. Cytochemic characteristics of the neutrophilic HL-60 subline No. 14. (A) Biebrich-scarlet stain (original mag ×500; current mag ×200). Cells are completely negative for eosinophilic granules seven days after refeeding. (B) Myeloperoxidase stain (original mag ×250; current mag ×96). Almost all cells are strongly positive with rather fine granular pattern. (C) Cyanide-resistant myeloperoxidase stain (original mag ×500; current mag ×96). The reaction is almost completely inhibited, indicating that they have neutrophil-type enzyme.

on day 7) in the absence of GM-CSF; the cloning efficiency was 21% in P-25, 5% in No. 3,[4] 7% in No. 3,[5] 10.3% in No. 14, and 4.6% in No. 8. All sublines also responded to GM-CSF (1 nmol/L), increasing the cloning efficiency by 236%, 440%, 380%, 270%, and 482%, respectively, as well as the size of the colonies (400 ~ 1,000 cells). Cytochemical analysis of individual colonies formed by these sublines was undertaken. In each of Nos. 3[4] and 3,[5] all of 100 colonies analyzed showed eosinophilic differentiation in both GM-

CSF–containing and control cultures. Eosinophil content in each colony varied widely from 10% to 100%. Nos. 8 and 14 did not form eosinophilic colonies. Thus, the tendency toward single-lineage differentiation was apparently inherited by progeny cells in all sublines.

Differentiation induction in single-lineage HL-60 sublines. As shown in Table 5A, the No. 3^4 eosinophilic subline manifested marked nuclear segmentation after one week of exposure to DMSO or retinoic acid (RA). The nuclear maturation, however, was paradoxically accompanied by the disappearance of eosinophilic granulation, as well as myeloperoxidase activity. In contrast, oxidative metabolism as detected by NBT dye reduction, was induced. The degrees of morphologic and metabolic changes were proportionate to the inducer concentrations. TPA was highly suppressive to the cell growth, but residual cells exhibited a marked morphologic shift to macrophage-like cells characterized by enlargement of cell sizes with increasingly low nuclear-cytoplasmic ratio and vacuolation. These cells also tended to lose eosinophilic granules and myeloperoxidase, but expressed weak Es-b activity, which is consistently strong in normal macrophages. In contrast, GM-CSF provided further nuclear maturation without any change in eosinophilic granulation and enzymatic pattern. No neutrophilic or monocytic differentiation was induced by GM-CSF. Subclone No. 3^5 exhibited similar findings (data not shown).

The No. 14 neutrophilic subline also showed marked nuclear segmentation after exposure to DMSO and RA (Table 5B). This nuclear maturation was accompanied by an increased rate of NBT dye reduction, but myeloperoxidase and Es-chl activities were markedly reduced. TPA was also markedly inhibitory of cell growth. The residual cells showed macrophage-like metamorphosis which was accompanied by a moderate degree of Es-b expression. Interestingly, eosinophilic cells appeared as a minor population in the culture with the lowest concentration (10^{-8} mol/L) of TPA. This was confirmed by the increased number of positive cells stained for cyanide-resistant myeloperoxidase. Eosinophilic differentiation, however, could not be induced by GM-CSF. The degree of nuclear segmentation was proportionate to the concentration of GM-CSF. Monocytic differentiation was also enhanced by GM-CSF. The degree of enhancement seemed to be inversely related to the concentration of GM-CSF. Interestingly, many cells showed double staining for Es-b and Es-chl. The No. 8 subline exhibited a similar pattern of induction (data not shown).

In the HL-60 parent line (low passage P-25), the typical induction pattern was obtained, ie, marked nuclear maturation by DMSO and RA, and macrophage-like transformation by TPA (Table 5C). As observed in single-lineage sublines, myeloperoxidase and Es-chl activities were slightly reduced by DMSO and RA. The lowest concentration of TPA also enhanced eosinophilic differentiation. GM-CSF enhanced nuclear segmentation and monocytic differentiation was also induced. Monocytic cells exhibited intense Es-b activity. Eosinophilic differentiation was also enhanced by GM-CSF. NBT dye reduction rate was increased after exposure to all chemical inducers and GM-CSF.

Alkaline phosphatase activity which is specifically expressed in mature neutrophils could not be detected in any differentiation-induction experiment, including that with GM-CSF.

DISCUSSION

Although the original report on the cytologic features of HL-60 cells and the fresh leukemic cells did not mention the presence of eosinophils,[2] we have confirmed the observations of others indicating constitutive eosinophilic differentiation.[13-16] Monocytic differentiation of HL-60 can also be induced by purified biosynthetic human GM-CSF.[5] Among normal myeloid progenitors, CFU-GM and eosinophilic progenitors (CFU-EO) have been regarded as distinct subpopulations which, as a rule, do not appear as mixed colonies.[24-27] The stem cells of HL-60 therefore possess multipotential capacity in cellular differentiation involving at least the three myeloid lineages cited, and therefore are not equivalent to normal CFU-GM,[11,12] of which potentiality is restricted to neutrophilic and monocytic lineages.

Primary subcloning of the P-50 parent line (with higher eosinophil content than early passage P-29) led to sublines that were of generally mixed neutrophilic-eosinophilic type. However, two pure neutrophilic subclones (Nos. 8 and 14) were obtained. Secondary subcloning of No. 3 with the highest eosinophil content (78%) led to many eosinophilic lines with extremely high eosinophil content and only one mixed eosinophilic/neutrophilic clone. These subcloned lines with single-lineage differentiation showed self-maintaining capacity in liquid culture for more than three months, and persistence of single-lineage differentiation. Moreover, the colonies formed by these subclones were no longer mixed. These observations suggest that in the HL-60 progenitor compartment, a progressive and irreversible commitment toward single-lineage differentiation is occurring; ie, the multipotent HL-60 stem cells are capable of becoming unipotent (or bipotent in the case of the neutrophilic subline) progenitors. These single-lineage HL-60 sublines (except No. 8) have growth characteristics in liquid culture and cloning efficiency in methylcellulose culture similar to their parent line. Their self-maintaining capacity indicates that the unipotent progenitors of these sublines have a self-renewal capacity similar to the multipotent progenitors of the parent line.

The differentiation-induction experiments with GM-CSF, a physiologic stimulator of CFU-GM and CFU-EO,[5] showed that it was no longer possible to induce neutrophilic or monocytic differentiation in the eosinophilic sublines and, conversely, eosinophilic differentiation in the neutrophilic sublines. GM-CSF induced monocytic differentiation only in the neutrophilic sublines and enhanced cellular maturation as evidenced by nuclear segmentation in both eosinophil and neutrophil sublines. These findings further support the view that the clonogenic cells of these single-lineage HL-60 sublines are unipotent committed progenitors. In contrast to the results with GM-CSF, the nonphysiologic inducers produced highly unbalanced expressions of various maturational features. For example, DMSO and retinoic acid markedly enhanced the nuclear segmentation in both types of single-lineage sublines, but this maturation was paradoxically associated with the disappearance of eosinophilic granules in the eosinophilic sublines, the reduction of Es-chl activity in the neutrophilic sublines, and the marked reduction of myeloperoxidase activity in both sublines. Oxidative metabolism, as assessed by the NBT dye reduction, was enhanced in the neutrophilic sublines and newly induced in the eosinophilic sublines by DMSO and RA. Low concentrations of TPA curiously induced some eosinophilic differentiation in the neutrophilic sublines, although GM-CSF did not. TPA caused the appearance of some macrophage-like cells in the eosinophilic sublines that did not show monocytic change in response to GM-CSF. Thus, functional and morphologic changes may be induced in the single-lineage leukemic HL-60 cells by treatment with phorbol esters.

Although it is necessary to investigate further the response of these cell lines to other physiologic stimulators (CSFs)[21,28] and a variety of differentiation inducers,[29-32] our present results suggest that the HL-60 parent line possesses many multipotent progenitors in the stem cell compartment, some of which show a commitment to single-lineage differentiation. Thus, there may be a hierarchical order in the HL-60 stem cell compartment. Further systematic subcloning of a larger number of mixed eosinophilic-neutrophilic subclones will clarify whether lineage commitment is a general phenomenon in the HL-60 stem cells, or restricted to a minor population of them.

Metcalf reported on a clonal analysis of eosinophilic differentiation in HL-60, including secondary plating of HL-60 colonies formed in agar. Among primary spontaneous colonies, he observed 3% to 7% eosinophilic colonies with distinct Luxol Fast blue positive granules. Three sequential reclonings of single colonies resulted in cells that could elaborate up to 36% eosinophil colonies. However, further subcloning experiments failed to generate a high proportion of eosinophilic colonies. Therefore, he speculated that the undifferentiated HL-60 cells have an ability to randomly differentiate toward the eosinophilic lineage at a low frequency. It is not clear whether colonies consisting wholly of eosinophils were subcloned as in our study. Fischkoff et al also did not obtain pure or highly eosinophilic sublines from HL-60.[14] The apparent discrepancy between their results and ours may be partly explained by the difference in the degree of spontaneous eosinophilic differentiation between the parent lines used. P-50 showed a much higher degree of spontaneous differentiation as well as a higher frequency of primary colonies with high eosinophil content. Also, Metcalf and Fischkoff et al employed a sequential recloning method, whereas we used a subcloning method. The latter method made it possible to analyze precisely the lineages of differentiation in a given subclone before further subcloning. Simultaneous cytochemic identification of neutrophilic and eosinophilic cells facilitated the efficient selection of single-lineage sublines in our study.

Subclones Nos. 3 and 14 were primarily developed as colonies in the methylcellulose culture with GM-CSF. However, the eosinophilic sublines Nos. 3^4 and 3^5 and the neutrophilic subline No. 8 were derived from the culture without GM-CSF. Therefore, the commitment of the multipotent HL-60 stem cells to the unipotent progenitors can proceed spontaneously and does not necessarily require GM-CSF. However, it will be of interest to clarify the role of GM-CSF in the modulation of the commitment process. Single-cell manipulation of HL-60 progenitors will facilitate such an analysis. We suspect that GM-CSF is capable of enhancing certain types of commitment since the present study showed that GM-CSF increases the frequency of eosinophilic colonies in the parent culture of HL-60 cells. Lu et al also observed a similar increase in the frequency of eosinophilic colonies using crude samples of GM-CSF.[13] Metcalf, however, did not see a consistent increase of eosinophilic colonies when stimulated by GM-CSFα or mitogen-stimulated mouse spleen-conditioned medium, both of which are known to stimulate eosinophil colony formation by normal human bone marrow cells.[21,33]

A second aspect of GM-CSF action is the enhancement, in every type of HL-60 cell line, of the growth rate in liquid cultures and the cloning efficiency in methylcellulose cultures. The increase in the colony size was also marked. Based on these observations, HL-60 cells should have binding proteins (receptors) for GM-CSF. We have identified such receptors in our laboratory using a binding assay with radioiodinated GM-CSF.[34] Availability of HL-60 sublines with single-lineage differentiation will greatly facilitate studies on the mechanism of action of a variety of CSFs.

The eosinophilic HL-60 sublines, although leukemic in nature, are the first established human eosinophil cell lines. The eosinophilic nature of a minor population of HL-60 cells was established by the extensive cytologic studies of Fischkoff et al.[15] They demonstrated cyanide-resistant myeloperoxidase and major basic protein in addition to other morphologic characteristics of eosinophils. These findings were confirmed in our study on the eosinophilic sublines. Various biologic activities or factors potentially stimulatory to eosinophils[35-37] can be investigated using these sublines. They may also be useful for immunologic studies on host defense against various kinds of parasites. It will be possible to obtain a large amount of pure material such as major basic protein,[38] which has recently been recognized as an important factor in allergy, especially in releasing histamine from mast cells and basophils as a nonlytic process.[39] It may also be possible to raise monoclonal antibodies specific for eosinophil surface antigens. Detailed analyses on the cell surface marker profile and cellular functions such as phagocytosis and killing of micro-organisms are now in progress.

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REFERENCES

1. Collins SJ, Gallo RC, Gallagher RE: Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270:347, 1977

2. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ruscetti F, Gallo R: Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood 54:713, 1979

3. Gallo R, Ruscetti F, Collins S, Gallagher R: Human myeloid

leukemia cells: studies on oncornaviral related information and in vitro growth and differentiation, in Golde DW, Cline MJ, Metcalf D, Fox CF (eds): Hematopoietic Cell Differentiation. Orlando, Fla, Academic Press, 1978, p 335

4. Lusis AJ, Koeffler HP: Action of granulocyte-macrophage colony-stimulating factors: studies using a human leukemic cell line. Proc Natl Acad Sci USA 77:5346, 1980

5. Tomonaga M, Golde DW, Gasson JC: Biosynthetic (recombinant) human granulocyte-macrophage colony-stimulating factor: Effect on normal bone marrow and leukemia cell lines. Blood 67:31, 1986

6. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC: Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci USA 75:2458, 1978

7. Rovera G, Santoli D, Damsky C: Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci USA 76:2779, 1979

8. Rovera G, Olashoshaw N, Meo P: Terminal differentiation in human promyelocytic leukaemic cells in the absence of DNA synthesis. Nature 284:69, 1980

9. Breitman TR, Salonick SE, Collins SJ: Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. Proc Natl Acad Sci USA 77:2936, 1980

10. Tarella C, Ferrero D, Gallo E, Pagliardi GL, Ruscetti FW: Induction of differentiation of HL-60 cells by dimethyl sulfoxide: evidence for a stochastic model not linked to the cell division cycle. Cancer Res 42:445, 1982

11. Koeffler HP: Induction of differentiation of human myelogenous leukemia cells: therapeutic implications. Blood 62:709, 1983

12. Koeffler HP, Yelton L, Prokocimer M, Hirji K: Study of differentiation of fresh myelogenous leukemic cells by compounds that induce a human promyelocytic leukemic line (HL-60) to differentiation. Leukemia Res 9:73, 1985

13. Lu L, Broxmeyer HE, Pelus LM, Andreeff M, Moore MAS: Detection of Luxol Fast blue positive cells in human promyelocytic leukemia cell line HL-60. Exp Hematol 9:887, 1981

14. Metcalf D: Clonal analysis of the response of HL60 human myeloid leukemia cells to biological regulators. Leukemia Res 7:117, 1983

15. Fischkoff SA, Pollak A, Gleich RJ, Testa JR, Misawa S, Reber TJ: Eosinophilic differentiation of the human promyelocytic leukemia cell line HL-60. J Exp Med 160:179, 1984

16. Fischkoff SA, Condon M: Switch in differentiative response to maturation inducers of human promyelocytic leukemia cells by prior exposure to alkaline conditions. Cancer Res 45:2065, 1985

17. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenburg DP, Jones SS, Brown EL, Hay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM: Human GM-CSF: Molecular cloning of the cDNA and purification of the natural and recombinant proteins. Science 228:810, 1985

18. Gasson JC, Weisbart RH, Kaufman SE, Clark SC, Hewick RM, Wong GG, Golde DW: Purified human granulocyte-macrophage colony-stimulating factor: Direct action on neutrophils. Science 226:1339, 1984

19. Li CY, Lam KW, Yam LT: Esterases in human leukocytes. J Histochem Cytochem 21:1, 1973

20. Konwalinka G, Glaser P, Odavic R, Bogusch E, Schmalzl F, Braunsteiner H: A new approach to the morphological and cytochemical evaluation of human bone marrow CFUc in agar cultures. Exp Hematol 8:434, 1980

21. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Separation of functionally distinct human granulocyte-macrophage colony stimulating factors. Blood 54:614, 1979

22. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. Amer J Clin Pathol 55:283, 1971

23. Zucker-Franklin D, Grusky G: The identification of eosinophil colonies in soft-agar cultures by differential staining for peroxidase. J Histochem Cytochem 24:1270, 1976

24. Shoham D, David EB, Rozenszajn LA: Cytochemical and morphologic identification of macrophages and eosinophils in tissue cultures of normal human bone marrow. Blood 44:221, 1974

25. Johnson GR, Dresch C, Metcalf D: Heterogeneity in human neutrophil, macrophage, and eosinophil progenitor cells demonstrated by velocity sedimentation separation. Blood 50:823, 1977

26. Dresch C, Johnson GR, Metcalf D: Eosinophil colony formation in semisolid cultures of human bone marrow cells. Blood 5:835, 1977

27. Dao C, Metcalf D, Bilski-Pasquier G: Eosinophil and neutrophil colony-forming cells in culture. Blood 50:833, 1977

28. Abboud CN, Brennan JK, Barlow GH, Lichtman MA: Hydrophobic adsorption chromatography of colony-stimulating activities and erythroid-enhancing activity from the human monocyte-like cell line, GCT. Blood 58:1148, 1981

29. Chiao JW, Freitag WF, Steinmetz JC, Andreeff M: Changes of cellular markers during differentiation of HL-60 promyelocytes to macrophages as induced by T-lymphocyte conditioned medium. Leukemia Res 5:477, 1981

30. Olsson I, Sargalasseril M, Breitman TR, Gallo RC: Isolation and characterization of a T lymphocyte-derived differentiation inducing factor for the myeloid leukemic cell line HL-60. Blood 63:510, 1984

31. Munroe D, Sugiura M, Griffin J, Kufe D: Effect of ara-A on differentiation and proliferation of HL-60 cells. Leukemia Res 8:355, 1984

32. Koeffler HP, Ranyard J, Yelton L, Billing R, Bohman R: γ-interferon induces expression of the HLA-D antigens on normal and leukemic human myeloid cells. Proc Natl Acad Sci USA 81:4080, 1984

33. Metcalf D, Cutler RL, Nicola NA: Selective stimulation by mouse spleen conditioned medium of human eosinophil colony formation. Blood 61:999, 1983

34. Gasson JC, Kaufman SE, Weisbart RH, Tomonaga M, Golde DW: High-affinity binding of granulocyte-macrophage colony-stimulating factor to normal and leukemic human myeloid cells. Proc Natl Acad Sci USA 83:669, 1986

35. Ruscetti FW, Cypess RH, Chervenick PA: Specific release of neutrophilic- and eosinophilic-stimulating factors from sensitized lymphocytes. Blood 47:757, 1976

36. Bartelmez SH, Dodge WH, Bass DA: Differential regulation of spleen cell-mediated eosinophil and neutrophil-macrophage production. Blood 55:489, 1980

37. Enokihara H, Hamaguchi H, Sakamaki H, Hazama S, Saito K, Furusawa S, Shishido H: Specific production of eosinophil colony stimulating factor from sensitized T cells from a patient with allergic eosinophilia. Brit J Haematol 59:85, 1985

38. Wassom DL, Loegering DA, Solley GO, Moore SB, Schooley RT, Fauci AS, Gleich GJ: Elevated serum levels of the eosinophil granules major basic protein in patients with eosinophilia. J Clin Invest 67:651, 1981

39. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D: Cytotoxic properties of the eosinophil major basic protein. J Immunol 123:2925, 1979

A Collaborative, Double-Blind Randomized Study of Cetiedil Citrate in Sickle Cell Crisis

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We have recently completed a double-blind, placebo-controlled, noncrossover study, the goal of which was to determine whether cetiedil citrate (cetiedil) could affect the course of vaso-occlusive crises in sickle cell disease. Patients, who presented to the emergency room at least 4 but no more than 24 hours after the onset of a painful vasoocclusive crisis severe enough to require hospitalization, were considered candidates for the study. Each patient received either placebo or cetiedil at one of the following three dosages: 0.2, 0.3, or 0.4 mg/kg body weight. The assigned drug dosage was given as a 30 minute intravenous infusion every 8 hours for 4 consecutive days. A total of 67 patients was enrolled in the study. Cetiedil, at its highest dosage (0.4 mg/kg body weight), was found to be significantly superior to placebo both in reducing the number of painful sites present on all 4 treatment days and in shortening the total time in crisis. No serious adverse reactions were observed during the course of the study. We conclude that cetiedil, given at a dosage of 0.4 mg/kg body weight, is therapeutically advantageous for sickle cell crisis.

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AMONG the most troublesome sequelae of the substitution of valine for glutamic acid at the sixth position of the beta chain of the hemoglobin molecule are recurrent vaso-occlusive crises.[1,2] To date, no specific treatment that will terminate or prevent these episodes is available.[3,4] Urea and sodium cyanate showed promise during initial in vitro studies but were found to be either ineffective or toxic in subsequent clinical testing.[5-11] A wide variety of other agents has also been proposed, but none has as yet been proven effective in controlled clinical trials.[3,4,12-14]

Cetiedil citrate (cetiedil), an iminoester developed from 3-thienyl acetic acid, was originally used as a vasodilator for peripheral vascular diseases such as Raynaud's phenomenon and intermittent claudication.[15-17] Cabannes reported that cetiedil, when administered intravenously on an open basis, shortened the duration of acute sickle cell crisis.[18] Further interest in cetiedil as a potential therapeutic agent in sickle cell anemia came from the observation by several groups in the United States that the drug exhibited in vitro antisickling activity at micromolar concentrations.[19-22] This laboratory evidence, along with the Cabannes experience and a favorable safety profile, served as the impetus to perform a controlled, double-blind, randomized trial to test the safety and efficacy of cetiedil in the treatment of acute painful crisis in sickle cell anemia.

MATERIALS AND METHODS

Patients. Patients with a known history of sickle cell disease, either sickle cell anemia (SS), sickle cell-beta Thalassemia (SB^0 Thal, SB^+ Thal), or sickle cell-hemoglobin C disease (SC), entered into the study. Hemoglobinopathies were confirmed by electrophoresis at a central laboratory. In this study, sickle cell crisis was defined as the sudden onset of pain involving one or more sites (extremities, back, abdomen, chest) typical of the patient's usual crisis for which there was no other explanation. Atypical isolated chest or abdominal pain was not considered to be crisis pain. Each patient was 19 years of age or older, capable of giving informed consent, and in sickle cell crisis for at least four hours, but not longer than 24 hours. Admission to the study was limited to one crisis per patient.

Criteria for exclusion were pregnancy, women of childbearing potential, incarcerated persons, patients with a history of drug abuse or drug dependency, transfusion within 90 days preceding the trial, acute cerebrovascular accident, overt infection, renal failure with a serum creatinine concentration greater than 2 mg percent (0.18 mmol/L), clinical or roentgenographic evidence of pulmonary edema, glaucoma, urinary retention, or a history of high sensitivity to anticholinergic or atropine-like drugs.

All studies were approved by the appropriate institutional review process and informed written consent was obtained from each patient. Whenever possible, this consent was obtained prior to the onset of the studied crisis.

Study design. The study was a double-blind, multi-investigator trial conducted at five clinical centers. Patients were randomly assigned at a central data center to treatment with either placebo (0.9 mg/mL, normal saline) or cetiedil (0.2 mg/kg, 0.3 mg/kg, or 0.4 mg/kg body weight) in normal saline. Drug, active or placebo, was presented in identically appearing ampules. The randomization plan specified a coded ampule and a volume (dosage) for each patient. The investigators were aware of the volume of medication but were not aware of the ampule's contents. Each patient received an IV infusion of study medication every eight hours over a four-day treatment period for a total of 12 doses. Fluid replacement and analgesic therapy were standardized. Choice of analgesics was limited to parenteral meperidine hydrochloride (1.2 mg/kg) or the equivalent of morphine sulfate for severe pain, and oral acetaminophen (300 mg) with codeine phosphate (30 mg) for moderate pain. Analgesia was not given within two hours before or after the beginning of medication infusion.

Determinants of efficacy and safety. Effectiveness of the study medication was examined by assessing pain intensity, number of

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painful sites, duration of crisis, the need for analgesic medication, and the investigator's overall evaluation of the patient's response to study medication.

For each patient, pain location and intensity were assessed and recorded on a modification of the standardized form utilized in earlier studies.[6] The scale for assessment of intensity was 0 = pain-free; 0.5 = residual tenderness or soreness; 1 = mild, movement does not increase pain; 2 = moderate, movement increases pain; 3 = severe, cannot move portions of body. Pain was evaluated by investigators and research nurses trained in assessing pain, just prior to therapy and at 1, 2, 4, and 6 hours after dosing. For pain intensity and number of painful sites, an average value was computed for each day of the study. Changes from baseline were analyzed for each treatment group, and each active drug group was compared to placebo.

Duration of crisis in this study refers to the time from the initiation of drug administration to the end of crisis. The sickle cell crisis was considered terminated when three of the following four criteria were satisfied: (1) absence of fever for at least 8 hours; (2) no progression of painful symptoms with absence of need for parenteral analgesics for at least 8 hours; (3) ability to walk without apparent difficulty; and (4) disappearance of pain symptoms and reduction of pain rating to ≤ 1 as evaluated on the scale as described above.

The average time in crisis for each of the three active drug groups was compared with that of the placebo group. Between-group differences in the proportion of patients requiring analgesic medication and the number of analgesic administrations were also examined.

To evaluate safety, clinically observed adverse reactions were recorded with regard to severity, onset, date, duration, frequency, drug relationship, and action taken. At the same time intervals as for pain assessment, vital signs (pulse, blood pressure, temperature, and respiration) were recorded. On days 1, 2, 3, 4, and 5 of the study and at the time of discharge, hemoglobin, hematocrit, complete blood count with differential, red cell indices, platelets, reticulocyte counts, prothrombin time, and fibrinogen levels were done. On days 1, 5, and day at discharge, electrocardiogram and additional laboratory tests, including fasting blood chemistries (urea nitrogen, glucose, SGOT, SGPT, alkaline phosphatase, uric acid, albumin, creatinine, total and direct bilirubin, cholesterol, triglycerides) and urinalysis, were performed. Finally, in each case an overall evaluation of the patient's response to study medication, taking into account both efficacy and toxicity, was performed by the investigators and rated as excellent, good, fair, or poor. A summary of the patient's course was also recorded. Patients were retained in the study for a minimum of 7 days.

Statistical methods. Continuous variables for patient characteristics and details of therapy were analyzed using a two-way analysis of variance. Discrete variables in these categories were analyzed using the Chi-square test or, for ranked data, the Kruskal-Wallis analysis of variance on ranks.[23] For crisis evaluation parameters, a two-way analysis of covariance was performed, using baseline as a covariate. Pairwise comparisons using t tests on adjusted treatment means were also made. For the variables, number of painful sites and pain intensity, a slope was calculated for each patient's collection of data points. Using the least squares regression technique, a two-way analysis of variance was performed on the slopes of the summary statistics. Using PIR multiple linear regression analysis program of the BMD-P statistical software (1983 edition), analysis of variance was performed on the slopes that were determined for all patients at each time point of the raw data. All within-treatment comparisons were accomplished by a paired t test on change from baseline. Global or overall evaluation and use of analgesic medication were analyzed by a two-way analysis of variance on ranks. The Mantel-Haenszel[24] procedure was used for comparisons of active drug to placebo for overall evaluations. For analgesic medication, comparisons were made using t tests on adjusted mean ranks.

Treatment by investigator interaction was evaluated for all parameters using a two-way analysis of variance. Since investigators did not know whether active drug or placebo was being administered to a given patient, but were aware of the volume of study medication given, study drug volume was included as a covariate in the statistical analysis of efficacy. It was subsequently determined that drug volume was not a significant source of variation in any of the analyses of key efficacy determinants, including number of painful sites, crisis duration, and global evaluation.

RESULTS

Sixty-seven patients were entered in the trial. Sixty-three patients were included in the analysis of efficacy. Four patients were found retrospectively to have high hemoglobin A concentration at baseline due to recent transfusions and were excluded. All 67 patients were included in the safety analysis. Baseline data for the 63 patients evaluated for efficacy are shown in Table 1. Fifty-seven per cent were male, 67% were between the ages of 20 and 29, and 87% had sickle cell anemia (SS). Statistical analysis revealed no significant differences among treatment groups for any of the baseline variables including pain intensity and number of painful sites.

Summary data for the change in average number of

Table 1. Baseline Variables

	Placebo	Cetiedil 0.2 mg/kg	Cetiedil 0.3 mg/kg	Cetiedil 0.4 mg/kg
Number of patients	16	16	18	13
Male	9	8	11	8
Female	7	8	7	5
Age (years)				
10–19	1	1	0	0
20–29	9	10	12	11
30–39	5	4	5	2
40–49	1	1	1	0
Mean	27.6	27.4	26.8	24.7
Weight (pounds)				
<120	3	8	3	3
120–140	5	4	4	6
140–160	6	2	7	3
>160	2	2	4	1
Mean	146.2	132.0	143.0	133.3
Hemoglobinopathies				
SS	13	14	16	12
SC	1	1	1	0
SB° Thal	0	0	1	0
SB^{+} Thal	2	1	0	1
Baseline variables				
No. of painful sites*				
Mean	3.56	3.31	4.17	4.31
Standard deviation	1.63	1.49	1.62	1.55
Pain†				
Mean	8.66	7.49	9.53	9.35
Standard deviation	5.06	3.24	4.67	4.18

*Scale: 0, None: 7, pain at all sites.

†Scale: 0, No pain at any site; 21, severe pain at all sites.

painful sites during the study are presented in Fig 1. The paired *t* within-group analyses of change from baseline documented significant improvement from baseline on all four days for both the middle dose (0.3 mg/kg) and high dose (0.4 mg/kg) of cetiedil ($P \leq 0.02$), while improvement from baseline in the low dose (0.2 mg/kg) and placebo groups was significant only on day 4. When treatment groups were compared to placebo (utilizing the baseline score as a covariate), only the high dose of active drug, cetiedil 0.4 mg/kg, was significantly superior ($P < 0.05$) on all four treatment days. These results for the 0.4 mg/kg dose compared with placebo were corroborated by analysis of the slopes calculated by using either the summary statistics for each patient ($P < 0.05$) or the raw data for each patient at each time point ($P < 0.001$).

In Fig 2, the influence of cetiedil on average pain intensity is seen. For within-group analysis of average pain, only the placebo group was not significantly improved on day 1: all treatments showed significant improvement on each day thereafter ($P < 0.02$). Comparisons of active drug to placebo (using baseline score as a covariate) indicated improvement in average pain intensity of statistical significance for the 0.3 mg/kg dose group on days 1 and 2 ($P = 0.02$). Cetiedil was not significantly superior to placebo at any doses upon

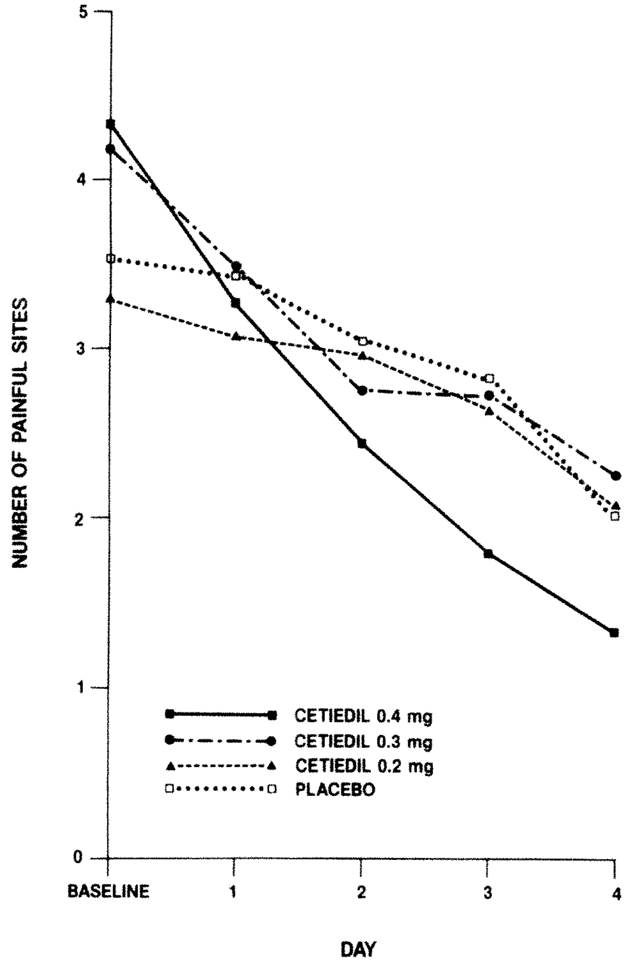

Fig 1. The raw means of painful sites for each treatment day are noted. The maximum number of painful sites were 7. Comparing differences from baseline for each patient using paired *t* test, both the 0.3 mg/kg and the 0.4 mg/kg cetiedil citrate treatments showed significant decrease from baseline in the average number of painful sites on each treatment day ($P \leq 0.02$). Including the baseline score as a covariate, compared with placebo, cetiedil citrate 0.4 mg/kg significantly decreased the average number of painful sites on each of the 4 treatment days (one-sided $P \leq 0.05$). Greater significance was noted upon analysis of the slopes of each patient's data reduced to one summary statistic ($P < 0.001$) or analysis of the slopes of unsummarized raw data for each patient ($P < 0.001$).

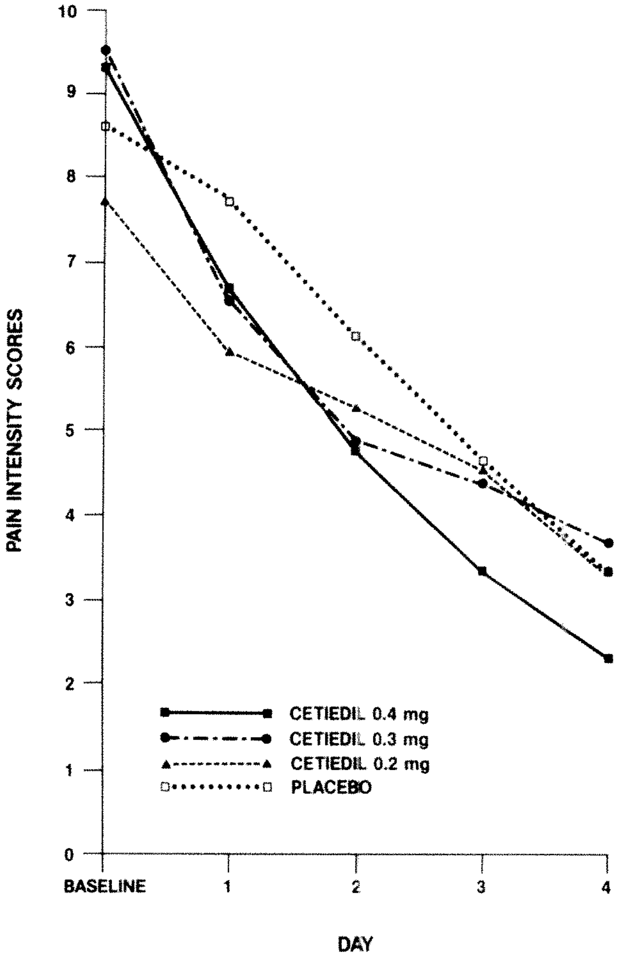

Fig 2. Average pain intensity depicted here is an average of pain assessments performed just prior to dosing and at 1, 2, 4, and 6 hours after dosing for each day of administration of study medication. In an attempt to quantify pain, a numerical value was assigned to each category, ranging from 0 representing no pain to 3 representing severe pain (see Methods). All treatments showed significant ($P \leq 0.05$) changes from baseline on all days except for placebo, which did not show a significant change on day 1. The raw means of 0.4 mg/kg groups showed a decrease in average pain intensity on all 4 days when compared with placebo. Cetiedil at the 0.4 mg/kg dose was not statistically superior to placebo as determined by either analysis of the adjusted means or the slopes of summary statistics for each patient. Analysis of slopes determined from multiple linear regression analysis of raw data for each patient at each time point indicated improvement in the 0.4 mg/kg dose when compared with placebo ($P < 0.01$).

analysis of the slopes of summary statistics for each patient. Analysis of the slopes calculated by multiple linear regression analysis of raw data for each patient at each time point indicated improvement in pain intensity by the 0.4 mg/kg dose when compared with placebo ($P < 0.01$).

Figure 3 shows results of statistical comparisons between cetiedil and placebo for duration of crisis (time from first administration of study medication to end of crisis). Cetiedil dosages of 0.3 mg/kg and 0.4 mg/kg were significantly superior to placebo ($P \leq 0.05$). There was no significant correlation between the degree of shortening of crisis after administration of study medication and the length of time in crisis within the 4 to 24 hour time period prior to treatment.

No significant differences between any of the cetiedil groups and placebo could be detected in either the proportion of patients requiring parenteral analgesic medication or the total number of doses given. Meperidine was the agent used greater than 95% of the time.

No serious adverse reactions were observed. The incidence of adverse effects (Table 2) was similar for the cetiedil 0.3 mg/kg, cetiedil 0.4 mg/kg, and placebo groups, and significantly lower ($P \leq 0.04$) for the cetiedil 0.2 mg/kg group. The most commonly reported adverse effects were headache and nausea/vomiting, for which no treatment or dose relationship was apparent, and dry mouth, which was noted only in the middle- and high-dose cetiedil groups.

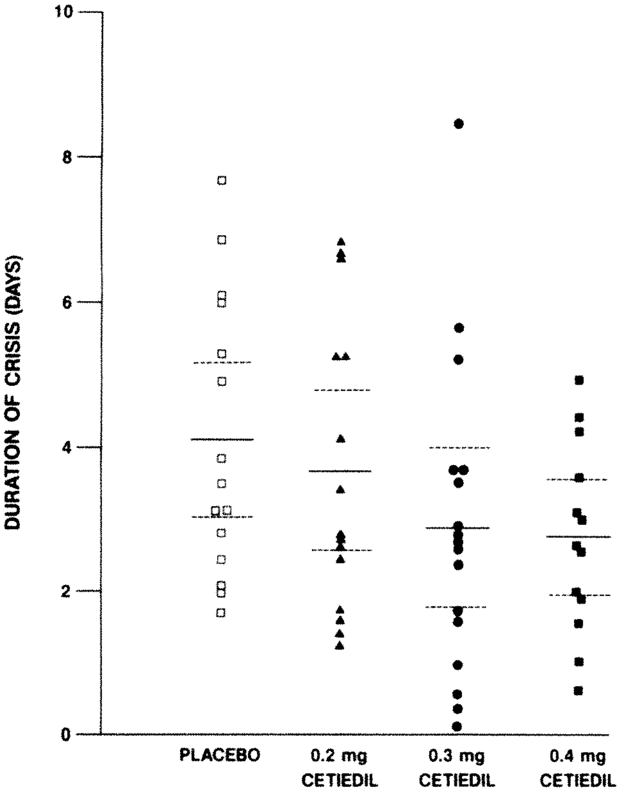

Fig 3. Shown here is a scattergram of the duration of crisis in days (the time from initiation of test medication to the end of crisis) for each study group. Cetiedil dosages of 0.3 mg/kg and 0.4 mg/kg were significantly superior to placebo $P \leq 0.05$. The solid and interrupted lines represent the means (——) and 95% confidence limits (····), respectively.

Table 2. Adverse Reactions by Treatment Group

		Number of Reports		
Adverse Reactions	Placebo	Cetiedil 0.2 mg/kg	Cetiedil 0.3 mg/kg	Cetiedil 0.4 mg/kg
Dry mouth	—	—	2	4
Nausea	3	1	1	1
Vomiting	3	—	2	—
Headache	4	—	5	2
Drowsiness	—	—	2	1
Constipation	1	—	1	—
Pruritus	1	—	1	—
Chills	—	—	1	—
Myalgia	—	1	—	—
Hypotension	—	—	—	1
Wenkebach arrhythmia	—	1	—	—
Migraine	1	—	—	—
Junctional rhythm and PVCs	1	—	—	—
Flatulence	—	—	1	—
Hematemesis	—	—	1	—
Anorexia	—	1	—	—
Abdominal pain	1	—	—	—
Dizziness	—	—	—	1
Sweating	—	—	—	1
Dysuria	—	—	1	—
Total no. of reactions	15	4	18	11
Total pts. with reactions	8	2	9	8
Total no. of pts. studied	18	17	18	14

Few laboratory variables were significantly different with cetiedil treatment when compared with placebo. The red blood cell count and hematocrit decreased significantly from baseline ($P \leq 0.05$) with all treatment regimens except the 0.4 mg/kg cetiedil dose. Analyses of electrocardiograms revealed a statistically but not clinically significant difference in the P-R interval between the middle-dose cetiedil group and placebo on day 5 but the effect was not dose-related since it was not apparent in the higher dose group.

Taking into account both efficacy and safety considerations, investigators rated the patient's overall response to treatment as excellent, good, fair, or poor (Table 3). The difference in the mean scores between the high dose of cetiedil and placebo was significant ($P < 0.01$). Excellent or good responses were recorded for 85% of patients who received cetiedil 0.4 mg/kg, 56% of patients who received cetiedil 0.3 mg/kg, 56% of patients who received cetiedil 0.2 mg/kg, and 25% of patients who received placebo. No

Table 3. Overall Evaluation Distribution by Treatment

	Overall Evaluation					
Study Drug	Excellent	Good	Fair	Poor	N	Mean*
Placebo	2	2	8	4	16	2.13
Cetiedil 0.2 mg/kg	4	5	3	4	16	2.56
Cetiedil 0.3 mg/kg	6	4	5	3	18	2.72
Cetiedil 0.4 mg/kg	7	4	2	0	13	3.38†

*Scale for means: 4, Excellent; 3, Good; 2, Fair; 1, Poor.

†Cetiedil 0.4 mg/kg was significantly superior to placebo ($P < 0.01$).

significant treatment by investigator interactions was noted in the efficacy and safety analyses.

DISCUSSION

This investigation demonstrates a measurable improvement in the course of the vaso-occlusive crisis in patients treated with cetiedil. Compared to placebo, the highest dose of cetiedil tested (0.4 mg/kg total body weight) was associated with significant reduction in the number of painful sites on all treatment days and a shortening of the time in crisis after receiving the study medication. The investigators' global evaluations were excellent or good in 85% of the patients receiving the highest dosage of cetiedil, compared with 25% of the patients receiving placebo.

The safety of the intravenous administration of cetiedil was also demonstrated in this study, as only minimal side effects were observed. The only side effect that appeared to have a dose-dependent relationship was dry mouth, an expected result because of cetiedil's known anticholinergic activity. In order to determine whether dry mouth might have unblinded the study, the data were reanalyzed. It appears that this side effect had no such influence with respect to reduction in painful sites and duration of crisis. The overall or global evaluation in the mid- and high-dose cetiedil groups was better for those with, than without, dry mouth. However, the overall evaluations for the patients who received active drug but did not have dry mouth remained significantly superior to those of placebo patients.

The results of this study and in vitro studies with cetiedil are noteworthy for two principal reasons. This study demonstrates the drug's potential usefulness as an effective and safe therapy for the painful crisis of sickle cell disease. A beneficial effect, occurring after the initiation of vaso-occlusion, has not been noted in controlled double-blind antisickling trials of other agents.

Second, in vitro studies with cetiedil suggest that factors involved in the pathogenesis of painful crisis can be influenced by approaches independent of modification of the abnormal hemoglobin molecule. Unlike cyanate or urea, cetiedil does not increase the solubility or oxygen affinity of hemoglobin S.[19,20] Recent studies have suggested that cetiedil's antisickling activity might be due to the ability of the drug to alter erythrocyte membrane permeability. Sickling is influenced by the permeability of the red cell membrane primarily because the rate of hemoglobin S gelation is enhanced by cell dehydration and slowed by an increase in cell water content.[25-27] Calcium-induced potassium loss and cell dehydration, which have been observed in very dense sickle cells, are inhibited by cetiedil.[28] Alternatively, the drug has been shown to enhance passive sodium permeability, thus causing cell swelling.[29] Other possible contributing factors to cetiedil's in vivo activity include such pharmacologic actions as peripheral vasodilation,[30-32] calmodulin inhibition,[21] reduction in plasma fibrinogen concentration and blood viscosity,[33] and inhibition of platelet aggregation.[28]

At present, the use of cetiedil citrate for treatment of vaso-occlusive crises in sickle cell anemia is restricted to investigations like the one described in this paper. Data from this study, coupled with the ability of cetiedil to inhibit sickling in vitro, serve as a rationale to continue investigations of cetiedil as a treatment in sickle cell disease. The importance of incorporating the rigorous methodology of this study with objective criteria for evaluation in future trials cannot be overemphasized. Only if subsequent trials confirm the findings in this report, should cetiedil be made available to practicing physicians for management of vaso-occlusive crises in sickle cell anemia.

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We are indebted to the physicians and nurses in our emergency departments and clinical research units. We wish to acknowledge Dr Toshio Asakura and Dr Elias Schwartz for participation in formulating this clinical study and for verification of hemoglobinopathies in the central laboratory.

REFERENCES

1. Ingram VM: A specific chemical difference between the globins of normal human and sickle cell anemia hemoglobin. Nature 178:792, 1956

2. Diggs LW: Sickle cell crisis. Am J Clin Pathol 44:1, 1965

3. Dean J, Schechter AN: Sickle-cell anemia: Molecular and cellular bases of therapeutic approaches. N Engl J Med 299:863, 1978

4. Brewer GJ: Detours on the road to a successful treatment of sickle cell anemia. Perspect Biol Med 22:250, 1979

5. Nalbandian RM, Schulz G, Lusher JM, et al: Sickle cell crisis terminated by intravenous urea in sugar solutions—a preliminary report. Am J Med Sci 261:309, 1971

6. Cooperative Urea Trials Groups: Clinical trials of therapy for sickle cell vaso-occlusive crisis. JAMA 228:1120, 1974

7. Cooperative Urea Trials Group: Treatment of sickle cell crisis with urea in invert sugar: A controlled trial. JAMA 228:1125, 1974

8. Cerami A, Manning JM: Potassium cyanate as an inhibitor of the sickling of erythrocytes in vitro. Proc Natl Acad Sci USA 68:1180, 1971

9. Peterson CM, Cerami A: Therapy of sickle cell disease, an approach with sodium cyanate. Semin Hematol 11:569, 1974

10. Harkness DR, Roth S: Clinical evaluation of cyanate in sickle cell anemia. Prog Hematol 9:157, 1975

11. Peterson CM, Tsaris P, Ohnishi A, et al: Sodium cyanate-induced polyneuropathy in patients with sickle cell disease. Ann Int Med 81:152, 1974

12. Brewer GJ, Kruckeberg WC: The anti-calcium and erythrocyte membrane effects of zinc, and their potential value in the treatment of sickle cell anemia, in Development of Therapeutic Agents for Sickle Cell Disease. Amsterdam, North-Holland Publishing, 1979, pp 195–204

13. Maugh TH: Sickle cell (II): Many agents near trials. Science 211:468, 1981

14. Klotz JM, Haney DN, King LC: Rational approaches to therapy: Anti-sickling agents. Science 213:724, 1981

15. Linquette M, Fossati P, Luez G: Interet therapeutic d'une nouvelle medication antiischemique et vasoregulatrice: Le cetiedil. Lille Med 18:1302, 1973

16. Soeterboek AM, Scaf AHJ, Lammers W, et al: Human experience of cetiedil, a new vasodilator with anticholinergic properties. Eur J Clin Pharmacol 12:205, 1977

17. Barbe R, Amiel M, Pouzeratt B, et al: Evaluation of cetiedil (Stratene) in peripheral vasodilation. Clin Trials J 17:20, 1980

18. Cabannes R, Maron P, et al: Preliminary study of the effects of Cetiedil on acute symptoms of sickle cell anemia. Clin Trials J 18:114–127, 1981 (Presented at Symposium International sur la Maladie Vasculaire, Rome, February 18–19, 1977)

19. Benjamin L, Kokkini G, Peterson CM: Cetiedil: Its potential usefulness in sickle cell disease. Blood 55:265, 1980

20. Asakura T, Ohnishi ST, Adachi K, et al: Effect of cetiedil on erythrocyte sickling: New type of antisickling agent that may affect erythrocyte membranes. Proc Natl Acad Sci USA 77:2955, 1980

21. Brewer GJ, Bereza U, Mizukami J, et al: Drug action hypothesis: How membrane expansion, calmodulin inhibition, and sickle cell therapy relate, in Brewer GJ (ed): Progress in Clinical and Biological Research. New York, Liss, 1981, pp 187–207

22. Berkowitz LR, Orringer EP: Effect of cetiedil, an in vitro antisickling agent, on erythrocyte membrane cation permeability. J Clin Invest 68:1215, 1981

23. Kirk R: Experimental Design: Procedures for the Behavioral Sciences. Monterey, Calif, Brooks/Cole, 1968, pp 497–498

24. Mantel N: Chi-square tests with one degree of freedom: Extension of the Mantel-Haenszel procedure. J Am Stat Assoc 58:690, 1963

25. Sunshine HR, Hofrichter J, Eaton WA: Requirements for therapeutic inhibition of sickle haemoglobin gelation. Nature 275:238, 1978

26. Glader BE, Nathan DG: Cation permeability alteration during sickling: Relationships to cation composition and cellular hydration of irreversibly sickled cells. Blood 51:823, 1978

27. Clark MR, Guatelli JC, Mohandas N, Shohet SB: Influence of red cell water content on the morphology of sickling. Blood 55:823, 1980

28. Berkowitz LR, Orringer EP: Effects of cetiedil on monovalent cation permeability in the erythrocyte: An explanation for the efficacy of cetiedil on the treatment of sickle cell anemia. Blood Cells 8:283, 1982

29. Schmidt WF, Asakura T, Schwartz E: Effect of cetiedil on cation and water movement in erythrocytes. J Clin Invest 69:589, 1982

30. Boissier JR, Aurousseau M, Gindiulli JF, et al: Pharmacological findings on cetiedil. Arzneim-Forsch Drug Res 28:2222, 1978

31. Simaan JA, Aviado DM: A comparative study between the cardiovascular effects of cetiedil, a new vasodilator, and papaverine and aminophylline. J Pharmacol Exp Ther 198:176, 1976

32. Aviado DM: Drug action, reaction and interaction III. New peripheral vasodilators. Drug Info J 10:36, 1976

33. Savi L, Pola P: Study of the blood viscosity and fibrinogen as a guide to the treatment of peripheral arterial atherosclerosis. Minerva Medica 69:4401, 1978

Effects of Recombinant Human GM-CSF on Proliferation of Clonogenic Cells in Acute Myeloblastic Leukemia

By James D. Griffin, D. Young, F. Herrmann, D. Wiper, K. Wagner, and K.D. Sabbath

Proliferation of acute myeloblastic leukemia (AML) cells in vitro is limited in most cases to a small subset of blasts that have several properties of stem cells. These leukemic colony-forming cells (AML-CFU) generally require addition of exogenous growth factors for proliferation in agar or methylcellulose. These factors can be supplied by media conditioned by phytohemagglutinin-stimulated normal leukocytes or by CSF-secreting tumor cell lines. However, the exact factor or factors required for stimulation of AML-CFU growth have not been defined. We compared the AML-CFU stimulatory activity of a human recombinant GM-CSF with that of GCT-CM, Mo-CM, and the PHA-leukocyte feeder system in 15 cases of AML. In each of the 12 cases that required exogenous growth factors for maximum AML-CFU growth, recombinant GM-CSF could replace either GM-CSF or Mo-CM, and could partially replace the PHA-leukocyte feeder system. These results indicate that this GM-CSF is a growth promoter of AML-CFU in these culture systems.

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A SMALL population of leukemic cells from most patients with acute myeloblastic leukemia (AML) will proliferate in semisolid media to form blast colonies.[1-3] These leukemic colony-forming cells (AML-CFU) have several properties associated with hematopoietic stem cells: self-renewal potential, high thymidine suicide index, and the capacity to undergo at least limited, although abnormal, differentiation to nonproliferative cells.[4-7] Growth of AML-CFU from most patients requires exogenous colony-stimulating factors (CSFs),[1,2,8,9] and these are commonly supplied by the addition of media conditioned by lectin-activated T cells,[2] or media from various tumor cell lines including GCT,[11] Mo,[12] or 5637.[13] However, these media contain multiple hematopoietic growth factor activities[14,15] and the exact factor or factors necessary for AML-CFU proliferation have not been identified. This is largely due to the difficulty in obtaining pure human CSFs. The gene for one human CSF normally expressed by T lymphocytes has recently been cloned and expressed by Wong and colleagues.[16] The recombinant CSF produced by COS cells transfected with this GM-CSF gene stimulates growth of normal day 14 and day 7 colonies of granulocytes, monocytes, and eosinophils, and may therefore be the equivalent of the previously partially purified CSF-alpha.[17] The cloned gene has approximately 70% homology to the murine GM-CSF gene.[16] We tested the ability of this recombinant GM-CSF (r-GM-CSF) to substitute for standard conditioned media in AML-CFU assays. The results indicate that this factor is a growth promoter of AML-CFU from many patients with AML.

MATERIALS AND METHODS

Leukemic cells. Peripheral blood or bone marrow aspirate samples were obtained at diagnosis, and the leukemic cells recovered by Ficoll-diatrizoate density gradient sedimentation. Peripheral blood was used whenever possible to minimize the possibility of "contamination" of AML-CFU with normal CFU-GM because of the very low numbers of CFU-GM found in normal blood. Mononuclear cells were cryopreserved in 10% dimethylsulfoxide in the vapor phase of liquid nitrogen until use. The diagnosis of AML was established by morphology,[18] cytochemic staining, and surface market analysis using a panel of antimyeloid monoclonal antibodies.[19] All samples contained more than 80% blasts. The 15 cases in this study were selected from a larger group (55 cases) which were screened for growth of AML-CFU in one or more of the culture systems tested (see below). Twenty-eight (51%) had clonogenic cells, and the 15 cases reported here were selected only on the basis of availability of sufficient numbers of frozen cells for repeated testing. The leukemic cells were further enriched by rosetting with sheep erythrocytes to remove T cells, and by plastic adherence at 37 °C to remove monocytes. Depletion of T cells was monitored by immunofluorescent staining with anti-T3.

Clonogenic assays. (1) Double layer agar assay was performed in quadruplicate by a modification of the method of Pike and Robinson.[7,20] Sources of CSF were incorporated in a 0.5% agar (Noble Agar, DIFCO) underlayer (0.5 mL). Target leukemic cells ($1-5 \times 10^4$/0.5 mL) were placed in a 0.3% agar overlayer. The medium was Iscove's modified Dulbecco's minimal essential medium (IMDM, Grand Island Biologicals, Long Island, NY) containing 20% fetal bovine serum (FBS) and further supplemented with glutamine and penicillin-streptomycin. The cell number was adjusted in order to generate fewer than 100 colonies or clusters per culture. At 7 to 10 days of culture, the agar overlayers were removed from the underlayers by agitation and dried onto glass slides under filter paper. The dried agar overlayers were fixed in acetone-citrate-methanol and colonies stained in situ for chloroacetate esterase and alpha-napthylacetate esterase activities to identify cells of myeloid lineage.[21] Colonies were defined as aggregates of 20 or more cells, and clusters as 8 to 19 cells.[3] (2) Phytohemagglutinin-leukocyte feeder system (PHA-LFS) was performed as described by Lowenberg[9] except that the overlayer contained 0.8% methylcellulose in addition to target leukemic cells and phytohemagglutinin (PHA). The underlayer contained irradiated 5,000 R normal leukocytes in 0.5% agar and was prepared one day prior to the addition of the leukemic cells as described.[9] Assays were performed in quadruplicate, and colonies counted on an inverted microscope on days 7 to 10. For each case the overlayers from several cultures were pooled and the cells washed free of methycellulose. The pooled cells were than examined for T3+ cells by immunofluorescence to exclude the possibility that the PHA had caused T-cell colony growth (less than 2% T3+ cells in all cases).

Conditioned media. GCT-CM was obtained from Gibco. Mo-CM and recombinant GM-CSF (r-GM-CSF) were obtained from Dr Steven Clark, Genetics Institute, Cambridge, Mass. Single lots of

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all conditioned media were used throughout this study. The r-GM-CSF was obtained as medium conditioned by COS cells transfected with the P91023 (B) expression vector containing the GM-CSF gene as previously described.[16] Medium conditioned by COS cells transfected with the vector not containing the GM-CSF gene was used as a control ("mock-transfected" COS-CM), and contained no detectable CSF activity for normal CFU-GM or AML-CFU in pilot experiments. In some experiments, highly purified recombinant-GM-CSF (0.1 ng/mL) was used to stimulate AML-CFU. The CSF was purified as described[16] and was provided by Dr Steven Clark.

In order to use equivalent amounts of colony-stimulating activity for the AML-CFU assays, the CSFs to be tested in these experiments were compared using a quantitative microproliferation assay in which immature normal bone marrow myeloid cells (purified approximately 50-fold by an immune rosette technique)[22] were exposed to titrated amounts of conditioned media, and the subsequent extent of induction of DNA synthesis at 48 hours measured by determining incorporation of ^{3}H-TdR during a 4-hour pulse as previously described.[22] We have previously shown that the cells in this assay system respond to various media containing GM-CSF, but not to erythropoietin, interleukin-2, phytohemagglutinin, or pokeweed mitogen.[22] Briefly, 5×10^3 partially purified marrow progenitor cells (typically composed of 25% to 50% blasts and 40% to 65% promyelocytes) were cultured in 150 uL of RPMI 1640 medium (Gibco) containing 10% FBS and varying concentrations of GCT-, Mo-, or r-GM-CSF–conditioned media at 37 °C for 44 hours at which time luCi of ^{3}H-TdR (Schwartz-Mann) in 50 uL of the same medium was added. The cells were than harvested 4 hours later on an automatic cell harvester.

Anti-CSF antibody. A sheep antiserum that neutralized r-GM-CSF activity was prepared and supplied by Dr Steven Clark at Genetics Institute by immunizing with highly purified GM-CSF.[16] In pilot experiments, immune serum had a neutralizing capacity in excess of 1 mg purified GM-CSF per mL of serum. In the experiments described here, equal volumes of r-GM-CSF–containing conditioned media (1/100 dilution of COS cell–conditioned medium) were incubated with anti-CSF serum or preimmune serum (1/200 dilution) for 1 hour at room temperature followed by assay of AML-CFU stimulatory activity.

Determining the surface antigen phenotype of L-CFC. In order to compare the AML-CFU proliferating in different culture systems, the phenotype of the clonogenic cells was determined with a panel of 5 monoclonal antibodies previously shown to react with some or all AML-CFU from different AML patients.[21] 1×10^6 leukemic cells were incubated with 100 uL of monoclonal antibody (ascites fluid diluted 1:100 in MEM containing 5% FBS) for 30 minutes at 4 °C, washed once, and further incubated with 100 uL of rabbit complement (1:6 dilution in MEM of rabbit serum, Pel-Freeze, Rogers, Ariz) for 90 minutes at 37 °C. The cells were then washed twice and the remaining number of viable clonogenic cells determined in each of the assay systems described above. The monoclonal antibodies and their reactivity with normal colony-forming cells were anti-MY9 (CFU-GEMM, d14 and d7 CFU-GM)[23]; anti-HLA-DR (Ia) (antibody 9-49 kindly supplied by Dr Robert Todd, University of Michigan, Ann Arbor, Mich) (CFU-GEMM, d14 and d7 CFU-GM)[24]; anti-PM-81 (d14 and d7 CFU-GM); anti-AML-2-23 (d7 CFU-GM) (kindly supplied by Dr Edward Ball, Dartmouth Medical School, Hanover, NH)[21,25,26]; H1712 (d14 and d7 CFU-GM; CFU-GEMM have not been tested). Further details of the reactivity of this panel of antibodies with normal progenitor cells and AML-CFU have been published.[21] In a previous series of cases, Ia and MY9 antigens were found to be expressed on the majority of AML-CFU from 18/20 patients, PM-81 on 13/20, and AML-2-23 on 6/20. H1712 was not tested in that series of cases, but preliminary data in our laboratory have indicated reactivity with AML-CFU in 6/7 cases. All experiments included a control of leukemic cells not treated with antibody or C′, and another control aliquot treated with a nonbinding antibody (an antigamma interferon antibody, 3C11C8) plus C′. The lot of C′ used in these studies was preselected for low toxicity to CFU-GM, and no nonspecific toxicity on AML-CFU was observed. In some experiments the number of AML-CFU remaining following antibody and C′ treatment are reported as a percent of control (anti-3C11C8 plus complement). All experiments were set up in quadruplicate.

RESULTS

AML cases. Cells from 15 AML patients were available for testing. If cells were available from both peripheral blood and bone marrow, the peripheral blood cells were used in order to minimize any possibility of having a mixture of normal and leukemic colonies.[21] Blasts constituted more than 80% of cells in all samples (Table 1). The patients ranged in age from 2 to 76 years. Five cases were FAB M1 or M2, 6 cases were M4, 2 cases were M5, and one case was unclassified. No case had a history of preleukemia or myeloproliferative syndrome.

Comparison of colony-stimulating activity from different sources. The relative potencies of the CSF-containing conditioned media used in this study were compared using a microproliferation assay with partially purified normal bone marrow myeloblasts and promyelocytes.[22] The response of 5×10^3 of these cells (^{3}H-TdR incorporation) to varying concentrations of GCT-CM, Mo-CM, and r-GM-CSF in a representative experiment are shown in Fig 1. The maximum response obtained with r-GM-CSF was lower than that obtained with GCT-CM or Mo-CM in 4 of 4 experiments. Mixing experiments with r-GM-CSF and Mo-CM showed that this was not due to the presence of inhibiting factors in r-GM-CSF (data not shown). One explanation would be the presence of other CSFs (such as CSF-beta) in Mo-CM or GCT-CM.[14,17] Based on the data in Fig 1, all further experiments in this study were performed with 10% (v/v) GCT-CM, 10% (v/v) Mo-CM, and 1% (v/v) r-GM-CSF. In

Table 1. AML Cases

Case Number	Age/Sex	Source of Cells*	FAB Classification†	% Blasts
1	65M	BM	M4	95
2	29M	PB	M1	91
3	33M	PB	M5	90
4	64F	PB	M5	99
5	4F	PB	M4	99
6	50F	PB	UNC	95
7	10M	PB	M4	99
8	56M	PB	M1	93
9	61M	PB	M4	99
10	33F	BM	M2	90
11	76F	BM	M4	97
12	55F	BM	M4	83
13	69F	PB	M1	85
14	2F	BM	M4	99
15	5F	BM	M2	90

*BM, bone marrow; PB, peripheral blood.

†French-American-British Classification[18]; UNC, unclassified.

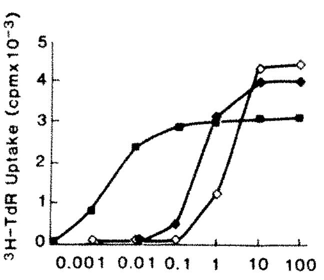

Fig 1. A comparison of CSF activity in various conditioned media. 5 × 10^3 partially purified normal bone marrow cells were cultured in 150 uL of RPMI 1640, 10% FBS, containing dilutions of GCT-CM (open diamonds), Mo-CM (closed diamonds), or recombinant GM-CSF (closed squares) for 44 hours. The cells were pulsed for 4 hours with ^{3}H-TdR, and incorporation measured. Background ^{3}H-TdR incorporation in this experiment was 422 cpm and has been subtracted. The results are expressed as the means of quadruplicate cultures. Standard error of the mean was less than ± 10% for each value.

order to be certain that these concentrations were also maximal for AML-CFU, cells from 2 AML cases were also cultured with higher concentrations of GCT-GM (20%), Mo-CM (20%), and r-GM-CSF (10%). In each case, maximum stimulation was achieved with 10%, 10%, and 1%, respectively (data not shown).

Effects of CSFs on AML-CFU. The effectiveness of GCT-CM, Mo-CM, and r-GM-CSF in the promotion of AML-CFU growth was studied in 15 patients with AML (Table 2). Conditioned media or control media (RPMI 1640 containing 10% FBS) was added to a double-layer agar culture. In the absence of added factors, in only one case were colonies formed (#5). Small clusters formed in several other cases, but in only 2 (#1, #3) were more than 50 clusters/5 × 10^4 observed. In 2 cases (#8, #13), small numbers of clusters formed in the absence of exogenous CSF which were not further stimulated by addition of the CM tested here. In 13 cases, cluster and/or colony growth was stimulated by GCT-CM, Mo-CM, and r-GM-CSF. In case #5 this stimulation was minimal due to the high level of autonomous proliferation. Colony formation in all cases except #5 was dependent on the addition of exogenous CSF. R-GM-CSF at the concentration tested provided stimulation equivalent to GCT-CM in all cases tested, and was equivalent to Mo-CM in all cases except #3 and #14. These results indicate that r-GM-CSF is identical to, or can replace, the growth factor(s) in GCT-CM or Mo-CM necessary for stimulation of AML-CFU growth in an agar assay system. Mock-CSF did not contain any AML-CFU stimulating activity (data not shown).

The leukemic nature of the colonies obtained was determined by morphology. For all experiments in agar, the cultures were dried onto glass slides, stained for ANAE and CAE activity, and counterstained with hematoxylin. The colonies and clusters were composed of immature myeloid cells in each case. In all 15 cases, the colonies stained for either ANAE, CAE, or both (data not shown). In each case, the staining pattern was homogeneous throughout all colonies; ie, either ANAE+, CAE−; ANAE−, CAE+; or ANAE+, CAE+. Mixtures of colony-staining patterns were not seen, as would be expected if normal CFU-GM were present in significant numbers. Colony morphology and cytochemic staining pattern was the same for each patient regardless of the source of CSF, further supporting the hypothesis that r-GM-CSF supports proliferation of the same AML-CFU as does Mo-CM or GCT-CM.

The PHA-stimulated leukocyte feeder system has been shown to support colony growth in a high fraction of AML patients.[9] Leukemic cells from 6 cases were also tested for growth in this system, and in 5 cases colonies or clusters were obtained. In 4 cases, r-GM-CSF provided equal or greater stimulatory activity (Table 2), while in 2 cases r-GM-CSF

Table 2. Growth of Clonogenic Cells in Different Culture Systems

	Cluster/Colony									
Case No.	Control*		GCT-CM		Mo-CM		r-GM-CSF		PHA-LFS	
1	592	0	620	110	810	129	767	136	310	160
2	0	0	231	9	216	8	205	6	75	2
3	90	0	725	9	1530	9	830	9	NT	NT
4	26	0	51	27	53	42	46	53	NT	NT
5	823	148	1150	210	956	198	1070	207	673	311
6	10	0	61	0	79	3	69	3	NT	NT
7	12	0	440	8	523	50	627	41	NT	NT
8	21	0	21	0	32	0	26	0	NT	NT
9	1	0	31	0	55	1	57	3	NT	NT
10	3	0	20	1	25	3	27	3	NT	NT
11	7	0	49	1	106	5	91	4	NT	NT
12	1	0	24	0	51	1	55	1	NT	NT
13	34	2	37	2	31	2	32	1	0	0
14	0	0	290	0	620	0	211	0	125	20
15	6	0	NT	NT	695	0	712	0	129	75

*Clusters (8–19 cells) or colonies (20 or more cells) per 5 × 10^4 cells (mean of 4 cultures, SE of mean was less than ± 25% in each case). Various sources of CSFs were incorporated in an agar underlayer (see Materials and Methods). Control was RPMI 1640 medium containing 10% FBS; GCT-CM and Mo-CM were used at a final concentration (v/v) of 10%, R-GM-CSF at 1%. In the PHA-LFS assay, irradiated PBL were incorporated in an agar underlayer, with leukemic cells and PHA in a 0.8% methylcellulose overlayer.

NT, not tested.

stimulated formation of only clusters while colonies and clusters were formed in the PHA-LFS assay.

There is limited information suggesting that some patients with AML may have more than one clonogenic cell either from different clones, or from different maturation stages of the same clone.[27] We therefore attempted to determine if the same clonogenic cell was growing in response to the different growth factors by analyzing the surface antigens expressed by AML-CFU with a panel of 5 cytotoxic monoclonal antibodies (Fig 2.)[21] In each of the cases tested the phenotype of the cells proliferating in response to r-GM-CSF was similar to that of the cells proliferating in response to GCT (6 cases) or PHA-LFS (2 cases). These results further support the possibility that r-GM-CSF is equivalent to the major AML-CFU growth factor in GCT-CM and possibly equivalent to that generated in the PHA-LFS.

Evidence that the AML-CFU stimulatory factor in COS cell–conditioned medium is GM-CSF. Leukemic cells from 4 cases were tested for response to highly purified GM-CSF[16] and for the effects of preincubation of COS cell–conditioned medium with a neutralizing sheep anti–GM-CSF antiserum (Table 3). Purified GM-CSF (0.1 ng/mL) had stimulatory activity equivalent to COS cell–conditioned medium (1% v/v). In addition, anti–GM-CSF partially or completely abrogated colony growth induced by COS cell–conditioned medium. These results suggest that the active factor in COS-CM is GM-CSF.

Table 3. Effects of Purified Recombinant GM-CSF and Anti-GM-CSF Antibody on AML Colony Formation

	AML-CFU*/5 × 10⁴ Cells				
Case No.	Control†	10% GCT	GM-CSF (COS-CM)	GM-CSF (Purified)	COS-CM + Anti–GM-CSF
2	0	255	298	277	12
7	14	348	369	352	63
14	21	422	410	387	109

*Colonies plus clusters produced in a 2-layer agar assay, enumerated at day 7.

†Control, medium alone; GM-CSF (COS-CM), 1% (v/v) medium conditioned by COS cells transfected with the GM-CSF genes; GM-CSF (purified), GM-CSF purified from COS-CM[16] used at 0.1 ng/mL; COS-CM (1% v/v) was incubated with an equal volume of 1:200 sheep anti–GM-CSF antiserum for 1 hr prior to adding to the agar cultures.

DISCUSSION

Proliferation of AML cells in vitro is limited in most cases to a small subset of blasts.[1-3] These cells are usually less mature than the rest of the leukemic cell population[7,28] and require exogenous growth factors for maximum colony growth in vitro, most often in the form of conditioned media from normal leukocytes or CSF-secreting tumor cell lines.[1-3,7-10] The recent cloning of a human GM-CSF gene[16] has provided an opportunity to assess the activity of the product of this gene, r-GM-CSF, as a promoter of AML-CFU growth in comparison to standard stimulatory factors. Fifteen AML cases were studied for response to this factor. AML-CFU from 12 cases required addition of exogenous factors for maximum AML-CFU growth, and in many cases, for any AML-CFU growth. In each of these cases, r-GM-CSF could replace either GCT- or Mo-CM, and in 6 cases tested, r-GM-CSF could partially or completely replace the PHA-leukocyte feeder system. Of the remaining cases, 2 formed a small number of clusters or colonies in the absence of any factors, and were not further stimulated by the addition of r-GM-CSF, Mo-CM, or GCT-CM. In the last case, the leukemic cells formed clusters and colonies autonomously with a high cloning efficiency (2%), and were minimally stimulated by any additional factor. Thus, for most cases which required a CSF for colony growth, r-GM-CSF could provide maximum stimulatory activity.

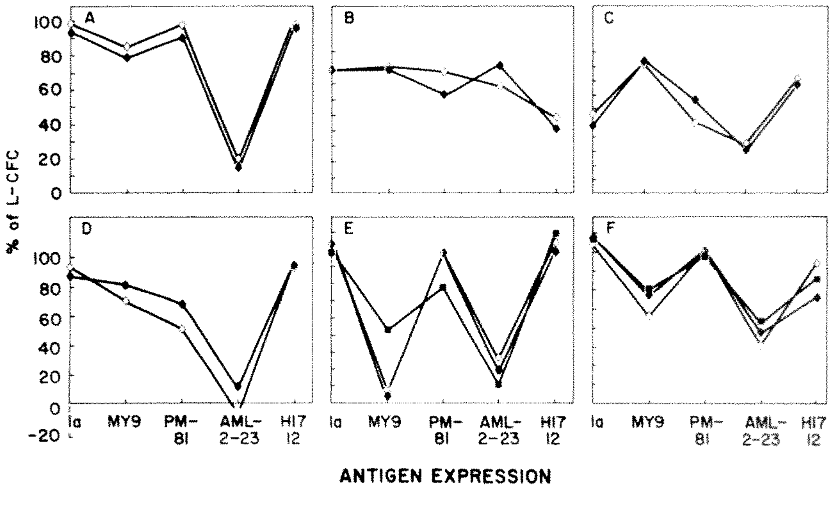

Fig 2. Surface antigen phenotype of clonogenic cells proliferating in response to various stimuli. Aliquots of leukemic cells from cases 3, 4, 15, 7, 2, and 1 (A–F, respectively) were treated with monoclonal antibody and complement and residual viable AML-CFU determined. The percentage loss of AML-CFU compared with control is a measure of the percent of AML-CFU expressing each antigen. Cells were then cultured in an agar assay with GCT-CM (open diamond) or r-GM-CSF (closed diamond). In 2 cases (E and F) cells were also cultured in the PHA-leukocyte feeder assay (closed squares).

Although a number of previous studies have documented the response of AML cells to conditioned media containing CSFs,[3,10,11] the active factor or factors in these media have not been previously defined. The various sources of CSFs that have been shown to be effective, including PHA-stimulated leukocytes, GCT-CM, placenta-CM, and Mo-CM, share the common property of containing GM-CSF activity, but most also contain other factors as well.[14,15,17] There have been some efforts to compare various conditioned media to determine the optimum source of AML-CFU stimulatory activity. Media conditioned by leukocytes alone are less effective than media conditioned by PHA-stimulated leukocytes (PHA-LCM).[3,9,29] Kubota compared PHA-LCM with Mo-CM and GCT-CM in both agar and methylcellulose cultures.[30] Although colonies from individual patients were sometimes better stimulated by one CM than another, overall there was no significant difference among the three, suggesting that there is at least one major AML-CFU growth factor common to all of these media. Taetle, however, found PHA-LCM to be superior to GCT-CM for many patients with AML and suggested either that PHA-LCM contains a higher concentration of an active AML-CFU growth factor, or a second growth factor lacking in GCT-CM.[29] Whether these differences are due to different concentrations of the same growth factors in various media or due to differences in growth factor requirements by individual leukemic clones has not been investigated. Also, there has not been any study of inhibitory factors in these various CMs, and it may be relevant to consider variations in inhibitors present in these media.

The finding in this study of several cases in which cluster and/or colony formation was "autonomous" merits further investigation to determine if this represents a degree of baseline CSF-independent cell proliferation, autocrine CSF secretion, or the presence of activated CSF-secreting normal cells.

Murine myeloid leukemia cells also generally require a CSF for in vitro proliferation, although continuous culture has led to the emergence of cell lines that no longer require exogenous factors.[8,31-35] Exposure of some of these cell lines such as M1 or WEHI-3 to conditioned media results in leukemic cell differentiation and may suppress self-renewal.[14,31] The active factor in inducing differentiation has been shown to be G-CSF, while GM-CSF and interleukin-3 (IL-3) stimulate proliferation without inducing differentiation.[15] Interestingly, human CSF-beta and murine G-CSF are cross-active in both species, and may bind to the same receptor.[15] The receptors for murine GM-CSF and IL-3 appear to be different than the G-CSF receptor. So far, a human equivalent of IL-3 has not been found, and if a human IL-3 exists, it will be of considerable interest to determine the sensitivity of AML-CFU to this factor. In the results presented here, r-GM-CSF provided maximum stimulation for most cases. This does not prove that there is only one factor that can stimulate AML-CFU, or that the major growth factor in GCT-CM or PHA-LCM is identical to r-GM-CSF. Careful comparisons of r-GM-CSF with other purified CSFs will be necessary to ultimately determine if this factor is the only CSF required to stimulate AML-CFU, or if it is one of several such factors. It should also be noted that in most studies clonogenic cells cannot grow from 30% to 50% of AML cases, and this suggests that additional synergistic factors or entirely different factors are required to maintain in vitro proliferation of leukemic cells in these cases. The recent report by Hoang of a blast colony-stimulating factor secreted by the bladder carcinoma cell line HTB9 is of interest in this regard, as preliminary data suggested that this factor could be separated on a Sephadex G-100 column from the active factor in PHA-LCM.[36] Finally, the possibility that there is a hierarchy of leukemic progenitor cells in some patients with AML needs further evaluation, as these cells may have distinctive CSF requirements.

REFERENCES

1. Moore MAS, Williams N, Metcalf D: In vitro colony formation by normal and leukemic human hematopoietic cells: Characterization of the colony forming cells. J Natl Cancer Inst 50:603, 1973

2. Dicke KA, Spitzer G, Ahearn MJ: Colony formation in vitro by leukemic cells in acute myelogenous leukemia with phytohemagglutinin as stimulating factor. Nature 259:129, 1976

3. Buick RN, Till JE, McCulloch EA: Colony assay for proliferative blast cells circulating in myeloblastic leukemia. Lancet 1:862, 1977

4. Minden MD, Till JE, McCulloch EA: Proliferative state of blast cell progenitors in acute myeloblastic leukemia (AML). Blood 52:592–600, 1978

5. Buick RN, Minden MD, McCulloch EA: Self-renewal in culture of proliferative blast progenitor cells in acute myeloblastic leukemia. Blood 54:95–104, 1979

6. Marie JP, Izaguirre CA, Civin CI, Mirro J, McCulloch EA: Granulopoietic differentiation in AML blasts in culture. Blood 58:670, 1981

7. Griffin JD, Larcom P, Schlossman SF: Use of surface markers to identify a subset of acute myeloblastic leukemia cells with progenitor cell properties. Blood 62:1300–1303, 1983

8. Burgess AW, Metcalf D: The nature and action of granulocyte-macrophage colony stimulating factors. Blood 56:947, 1980

9. Lowenberg B, Swart K, Hagemeijer A: PHA-induced colony formation in acute non-lymphocytic and chronic myeloid leukemia. Leuk Res 4:143, 1980

10. Francis GE, Berney JJ, Chipping PM, Hoffbrand AV: Stimulation of human hematopoietic cells by colony stimulating factors: Sensitivity of leukaemic cells. Br J Haematol 41:545, 1979

11. Brennan JK, DiPersio JF, Abboud CN, Lichtman MA: The exceptional responsiveness of certain human myeloid leukemia cells to colony-stimulating activity. Blood 54:1230, 1979

12. Golde DW, Quan SG, Cline MJ: Human T lymphocyte cell line producing colony-stimulating activity. Blood 52:1068, 1978

13. Welte K, Platzer E, Li L, Gabrilove JL, Levi E, Mertelsmann R, Moore MAS: Purification and biochemical characterization of human pluripotent hematopoietic colony-stimulating factor. Proc Natl Acad Sci 82:1526, 1985

14. Abboud CN, Brennan JK, Barlow GH, Lichtman MA: Hydrophobic adsorption chromatography of colony-stimulating activities and erythroid-enhancing activity from the human monocyte-like cell line GCT. Blood 58:1148, 1981

15. Nicola NA, Begley CG, Metcalf D: Identification of the human analogue of a regulator that induces differentiation in murine leukemic cells. Nature 314:625, 1985

16. Wong GG, Witek JS, Temple PA, et al: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

17. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54:614, 1979

18. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick H, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976

19. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF: Surface marker analysis of acute myeloblastic leukemia: Identification of differentiation-associated phenotypes. Blood 62:557, 1983

20. Pike BL, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76:77–84, 1970

21. Sabbath K, Ball ED, Larcom P, Davis RB, Griffin JD: Heterogeneity of clonogenic cells in acute myeloblastic leukemia assessed by surface marker analysis. J Clin Invest 75:746, 1985

22. Griffin JD, Sullivan R, Beveridge RP, Larcom P, Schlossman SF: Induction of proliferation of purified human myeloid progenitor cells: A rapid assay for granulocyte colony-stimulating factors. Blood 63:904, 1984

23. Griffin JD, Linch D, Sabbath K, Schlossman SF: A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. Leuk Res 4:521–534, 1984

24. Todd RF, Meuer SC, Romain PL, Schlossman SF: A monoclonal antibody that blocks class II histocompatibility-related immune interactions. Hum Immunol 10:23, 1984

25. Ball ED, Graziano RF, Shen L, Fanger MW: Monoclonal antibodies to novel myeloid antigens reveal human neutrophil heterogeneity. Proc Natl Acad Sci USA 79:5374–5378, 1982

26. Ball ED, Graziano RF, Fanger MW: A unique antigen expressed by myeloid cells and acute leukemia blast cells defined by a monoclonal antibody. J Immunol 130:2937–2941, 1983

27. Lowenberg B, Hagemeijer A, Swart K: Karyotypically distinct subpopulations in acute leukemia with specific growth requirements. Blood 59:641–645, 1982

28. Lange B, Ferrero D, Pessano S, Palumbo A, Faust J, Meo P, Rovera G: Surface phenotype of clonogenic cells in acute myeloid leukemia defined by monoclonal antibodies. Blood 64:693–700, 1984

29. Taetle R, Caviles A, Koziol J: Response of human myeloid leukemia cells to various sources of colony-stimulating activity and phytohemagglutinin-conditioned medium. Cancer Res 43:2350, 1983

30. Kubota K, Preisler HD, Sagawa K, Minowada J: Comparison between agar and methylcellulose cultures of human leukemia cells. Cancer Res 41:3052, 1981

31. Ichikawa Y: Differentiation of a cell line of myeloid leukemia. J Cell Physiol 74:223, 1969

32. Metcalf D: Clonal analysis of the action of GM-CSF on the proliferation and differentiation of myelomonocytic leukemia cells. Int J Cancer 24:616, 1979

33. Sachs L: Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. Nature 274:535, 1978

34. Schrader JW, Crapper RM: Autogenous production of a hemopoietic growth factor, persisting-cell-stimulating factor, as a mechanism for transformation of bone marrow-derived cells. Proc Natl Acd Sci 80:6892, 1983

35. Heard JM, Fichelson S, Sola B, Martial MA, Varet B, Levy JP: Multistep virus-induced leukemogenesis in vitro: Description of a model specifying three steps within the myeloblastic malignant process. Mol Cell Biol 4:216, 1984

36. Hoang T, McCulloch EA: Production of leukemia blast growth factor by a human bladder carcinoma cell line. Blood 66:748, 1985

In Vitro Release of Physically Separable Factors From Monocytes That Exert Opposing Effects on Erythropoiesis

By Laurie Feldman, Carl M. Cohen, and Nicholas Dainiak

In order to investigate the capacity of monocytes to release erythroid burst-promoting activity (BPA), we added media conditioned by homologous monocytes to both serum-free human and serum-restricted murine marrow culture. We found that soluble, membrane vesicle-free culture medium is a potent source of the growth factor. On the other hand, monocyte membranes or exfoliated plasma membrane vesicles elaborate a factor that inhibits erythroid burst formation by up to 100%. Inhibitory activity is expressed in a dose-dependent fashion over a wide range of concentrations (0.001 to 10 μg/mL) tested. Experiments with antilymphocyte plasma membrane IgG, which has been shown to neutralize both soluble and membrane-bound lymphocyte-derived BPA in human marrow culture, indicate that the expression of soluble BPA by monocytes is unaffected by these antibodies. Furthermore, while antimembrane IgG is capable of absorbing BPA from LCM supernatants, these antibodies are ineffective in removing BPA from MCM supernatants, suggesting that these two soluble growth factors may be antigenically distinct. Our findings indicate that while monocytes release soluble BPA, they are also a source of membrane-associated factors that exert inhibitory effects on erythropoiesis in vitro.

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DIFFERENTIATION of multipotential stem cells along the erythroid pathway is dependent on the hormone erythropoietin and on a group of cell-derived factors known collectively as erythroid burst-promoting activity, or BPA.[1,2] It has been suggested that BPA is important for proliferation of the early erythroid progenitor, the burst-forming unit—erythroid (BFU-E). BPA is derived from many mammalian mononuclear cell types that include the lymphocyte and monocyte,[3-5] and it is released into cell-free liquid culture media conditioned by such cells. We have previously shown that BPA is expressed by lymphocyte membranes and by membrane components that are shed into lymphocyte-conditioned medium (LCM).[6] To examine the capacity of monocytes to release erythropoietic growth regulators, we have now tested human and murine monocyte membranes and cell-free medium conditioned by monocytes for their ability to support BFU-E proliferation in homologous bone marrow culture. We have found that membrane vesicle-free supernatant from monocyte-conditioned medium (MCM) is a potent source of BPA. In contrast, monocyte plasma membranes and membrane vesicle-rich pellets obtained by high speed centrifugation of MCM suppress burst formation. Testing with antilymphocyte membrane antiserum suggests that monocyte-derived BPA may be antigenically distinct from soluble and membrane-associated BPA derived from lymphocytes. Our results showing the release of two physically distinct factors from monocytes with opposing regulatory effects on erythropoiesis in vitro may provide an explanation for monocyte concentration-dependent stimulation and suppression of burst formation.[7-9]

MATERIALS AND METHODS

Preparation of conditioned media and medium fractions. Conditioned media were prepared from unstimulated human peripheral blood or mouse spleen mononuclear cells as previously described.[6] Informed consent was obtained before phlebotomy of hematologically normal human volunteers in accordance with the Human Investigation Committee of St. Elizabeth's Hospital of Boston.

Briefly, peripheral blood was aspirated into serum-free MEM alpha medium (Gibco, Grand Island, NY) containing 20 U/mL preservative-free heparin and separated 2 × over Ficoll-paque (Pharmacia Fine Chemicals, Piscataway, NJ). Single-cell suspensions of spleen cells from Crl:CF1 BR mice were prepared similarly in serum-free alpha medium. Mononuclear cells at the interface were removed, washed 3 × and incubated at 5 × 10^6/mL at 37 °C, 5% CO_2, 90 minutes in plastic tissue culture dishes. Nonadherent cells were removed, washed 1 × and both adherent and nonadherent cells were incubated for 24 hr, at 37 °C, 5% CO_2 in serum-free MEM alpha medium supplemented with L-glutamine, penicillin, and streptomycin (Gibco). At this point, nonadherent cells contained >97% lymphocytes and adherent cells contained >85% monocytes by staining with nonspecific esterase or periodic acid Schiff reagent, and by morphologic identification on Wright's stained smears. In some cases, indomethacin (Sigma Chemical Co, St. Louis, Mo), 10^{-6} mol/L, was added to both adherent and nonadherent cells at the onset of incubation. Whole LCM and MCM were harvested, freed of cells by centrifugation at 500 × g, 10 minutes, and separated into supernatants and pellets by centrifugation at 40,000 × g, 30 minutes. The pellets were washed 1 × and resuspended to their original volume in serum-free alpha medium for testing in culture.

Preparation of lymphocyte and monocyte plasma membranes. Plateletpheresis residues were obtained from the Dana-Farber Cancer Institute, Boston. Mononuclear cell concentrates were prepared as described[6] and depleted of residual platelets by the method of Perper et al.[10] Mononuclear cells were suspended at 5 × 10^6/mL in serum-free alpha medium and incubated in plastic tissue culture flasks for 90 minutes (×2) or overnight at 37 °C, 5% CO_2. After nonadherent cells were decanted, the flasks were rinsed 2 × with fresh alpha medium, and adherent cells were removed by gentle scraping. Both nonadherent (>95% lymphocytes) and adherent (>85% monocytes) cells were washed 2 × with alpha medium and 3 × with PBS. Monocyte and lymphocyte plasma membranes were isolated by differential centrifugation and sucrose gradient fractionation, according to a modification of the method of Jett et al[11] as previously described.[12] Mouse (Crl:CF1 BR) spleen mononuclear cells were similarly separated into nonadherent (>97% lymphocytes) and adherent (>85% monocytes) populations, from which plasma membranes were prepared as for the human cells. Purified membrane fractions were stored at −70 °C in PBS.

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Preparation of antilymphocyte membrane antiserum. Antilymphocyte plasma membrane antisera were prepared as previously described.[12] Briefly, 50 to 100 μg of human lymphocyte plasma membrane protein emulsified in Freund's adjuvant (Sigma) were injected at multiple intradermal sites into each of three female New Zealand white rabbits at days 0, 14, and 21. Animals were bled 7 days following the last injection. Thereafter, animals received intravenous booster injections at monthly intervals and were bled 5 days after each injection. IgG fractions were obtained from selected postimmune as well as from preimmune sera by $(NH_4)_2SO_4$ fractionation, followed by DEAE cellulose column chromatography. Sera and/or IgG fractions were tested for crossreactivity with the immunizing antigen as well as with human LCM fractions.[12]

Assays for erythroid burst-promoting activity (BPA). Approximately 0.5 mL of spiculated human bone marrow were aspirated from the posterior iliac crests of hematologically normal volunteers. Informed consent and approval of an Institutional Review Board were obtained prior to each donation. The cells were placed in Eagle's minimal essential medium (MEM, Gibco) containing heparin and separated over Ficoll–paque. Mononuclear cells layering at the interface were separated, washed in alpha-medium and cultured at 6 × 10^4/mL in 0.8% methylcellulose. Cultures were established under serum-free conditions as described previously.[13] They were supplemented with purified human serum albumin, transferrin, ferric chloride, and 2.0 U/mL of sheep Step III erythropoietin (Connaught Laboratories, Ontario, Canada) or recombinant human erythropoietin (70,000 to 80,000 U/mg, Amgen Biologicals, Thousand Oaks, Calif). Test plates contained 10% (v/v) LCM or its fractions, MCM or its fractions, lymphocyte or monocyte plasma membranes, or NCTC-109 at final concentrations indicated in Figure legends. Cultures were maintained for 12 to 14 days, 37 °C, 5% CO_2 in humidified air, and scored for orange bursts (BFU-E–derived colonies) with a grid under an inverted microscope. Cloning efficiency varied between 15 and 75 bursts/10^5 light density marrow cells plated. BPA expressed by each addition was measured as burst formation relative to that in cultures containing NCTC-109 (100 percent stimulation).

Murine bone marrow cultures were established in serum-restricted (4.5%, v/v, fetal calf serum) fibrin clots as described previously.[6] Briefly, marrow cells were flushed from the tibias of healthy Crl:CF1 BR mice, separated over Ficoll-paque, washed and seeded at a final concentration of 5 × 10^4/mL. Erythroid bursts containing >49 benzidine-positive cells were scored after 8 days of incubation. The amount of BPA present in each addition was determined by comparing burst formation at 4.5% serum relative to that in cultures containing NCTC-109 (100% burst formation).

Immunoadsorption of BPA with antimembrane IgG. Human LCM and MCM supernatants, demonstrated to contain BPA in serum-free methylcellulose cultures, were incubated overnight at 4 °C with increasing amounts of either normal (preimmune) or antilymphocyte membrane IgG. An amount of Pansorbin (Calbiochem, Los Angeles, Calif) calculated to have an absorption capacity four-fold in excess of the maximum amount of IgG used was added and incubated for 15 minutes, 25 °C before bound IgG was removed by centrifugation. Absorbed CM supernatants (10% v/v) were then assayed for BPA in serum-free cultures as described above.

Statistical methods. The number of erythroid bursts formed was determined in each of four 125 μL clots or in 1 mL methylcellulose plates. The mean ± SEM of quadruplicate determinations for each test point in culture was calculated. Data sets were compared by the two sample rank tests of Wilcoxon and White.[14]

RESULTS

Effects of monocyte CM fractions on burst formation. LCM prepared from either human peripheral blood (Fig 1A) or murine spleen cells (Fig 1B) stimulated BFU-E proliferation relative to control cultures. Testing of LCM fractions in homologous marrow culture revealed that BPA was expressed by both LCM supernatants and vesicle-rich pellets. In contrast, MCM prepared from either of these tissues strongly inhibited burst formation (Fig 1C and D). Inhibitory activity was localized to vesicle-rich MCM pellets, while vesicle-free MCM supernatant from either source stimulated BFU-E proliferation.

In many cases, the net biologic effects of LCM and MCM fractions were nonadditive (see Fig 1). This is expected when the effects measured are at the right-hand (or maximal) side of the growth factor dose-response curve. Therefore, experiments aimed at defining the dose response of burst-enhancing and suppressor molecules were performed. When serial dilutions of murine LCM and MCM pellets were added to culture, dose-dependent membrane-vesicle stimulation and inhibition of BFU-E growth, respectively, were observed. Figure 2 shows that maximal BPA was expressed at 1 μg of lymphocyte vesicular protein/mL, and that burst suppression was eliminated by dilution of resuspended monocyte pellets to 0.005 μg vesicular protein/mL. Similar results were obtained when various concentrations of human LCM and MCM pellets were assayed in serum-free human marrow culture (data not shown).

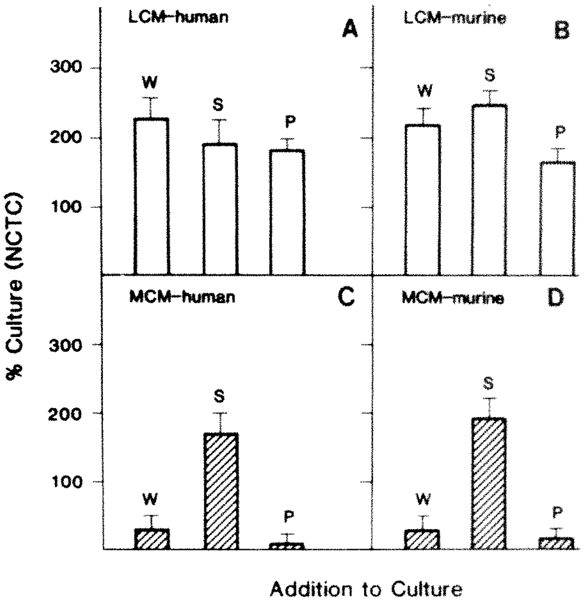

Fig 1. Effects of lymphocyte- (LCM) and monocyte-conditioned media (MCM) on human (A and C) and murine (B and D) homologous erythroid burst formation. Whole CM (W), supernatants (S), or pellets (P) were added at 10% (v/v) to serum-free methylcellulose cultures of human marrow[13] or serum-restricted (4.5% fetal calf serum) fibrin clot cultures of CF_1 mouse marrow[6] that contained 2.0 IU/mL sheep step III erythropoietin. Bursts appearing after 12 d (for human cultures) or 8 d (for murine cultures) of incubation were scored relative to control plates containing 10% (v/v) NCTC-109 (26 ± 2 bursts/dish for human; 60 ± 12 bursts/1.1 mL clot for murine). Mean stimulations ± SEM are shown for results in three human or four murine marrow cultures. 100% growth represents that in control plates containing NCTC-109. While P from LCM stimulate burst formation, those from MCM inhibit growth ($P < 0.05$ for each).

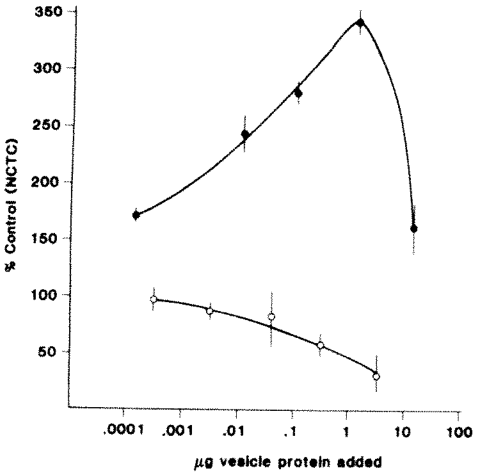

Fig 2. Effect of membrane-derived vesicles from LCM (●) and MCM (O) on burst formation in murine marrow culture. The translucent pellets from 70 mL CM were resuspended in a minimum volume of phosphate buffered saline (PBS, pH 7.4) for protein determination. Serial dilutions of vesicle protein were prepared in MEM alpha medium and added at 10% (v/v) to serum-restricted murine marrow cultures containing 2.0 IU/mL erythropoietin. Mean ± SEM percent burst formation in quadruplicates relative to growth in control cultures containing 10% (v/v) NCTC-109 is displayed. Control cultures contained 80 ± 10 bursts/1.1 mL clot. While 1 μg of vesicular protein from LCM augments BFU-E proliferation, similar amounts from MCM suppress burst formation ($P < 0.05$ for each).

Suppression of burst formation by monocyte membranes. We next examined the relationship between biologic activities of CM pellets and the plasma membranes from which they were derived. Results in Fig 3 indicate that human lymphocyte plasma membranes augment BFU-E proliferation in a dose-dependent fashion, while similar amounts of monocyte membranes reduce growth in a dose-dependent fashion by up to 75%. Similar results were obtained in tests with murine lymphocyte and monocyte membranes (data not shown).

Role of prostaglandins in mediating MCM activity. Since prostaglandins have been shown to alter the rate of erythroid burst and colony formation in vitro,[15-17] we next determined whether incubation of lymphocytes and monocytes with a prostaglandin synthesis inhibitor altered the release of erythroid burst stimulatory and/or inhibitory molecules. Figure 4 shows that addition of indomethacin to cells during incubation had no effect on growth promotion or suppression. This suggests that prostaglandin synthesis is not involved in CM effects that were measurable in culture.

Effects of antilymphocyte membrane IgG on BPA expression. Results of earlier experiments using rabbit antihuman lymphocyte membrane antiserum have indicated that antimembrane IgG reacts with multiple components of both LCM supernatants and LCM pellets.[12] When tested in human serum-free marrow culture, IgG fractions from these sera strongly inhibit BFU-E proliferation, an effect that is overcome by adding increasing amounts of LCM or LCM fractions to culture. While the direct addition of 10^{-2} μg/mL IgG completely inhibits BFU-E proliferation, this effect is overwhelmed by addition of 30% (v/v) LCM supernatant to culture (data not shown). In contrast, antimembrane IgG did not suppress burst formation in cultures containing as little as 10% (v/v) MCM supernatant. No IgG inhibitory activity was observed with normal (preimmune) IgG or with antimembrane IgG that was previously heated to 100 °C for 5 minutes before addition to culture.

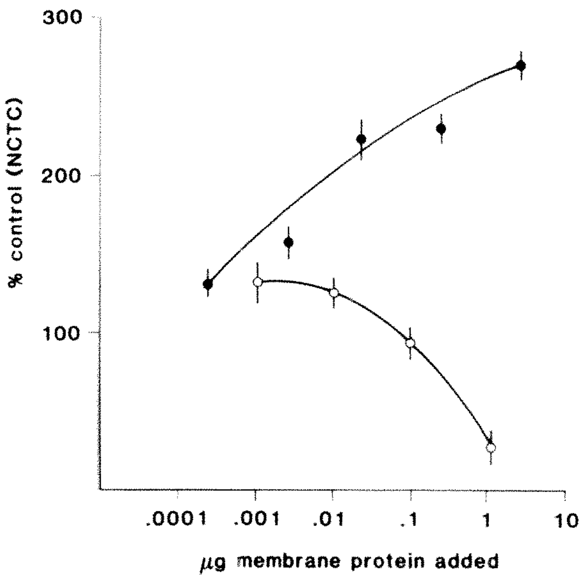

Fig 3. Activity expressed by lymphocyte (●) and monocyte (O) plasma membranes in culture. Plasma membranes were purified from human peripheral blood lymphocytes and monocytes as described.[12] Various amounts of membrane protein were added at 10% (v/v) to serum-free cultures of homologous bone marrow cells containing 2.0 IU/mL erythropoietin. Burst formation was assayed and quantitated as described in legend to Fig 1. Mean ± SEM percent burst formation in quadruplicates is displayed. Control cultures without added membrane protein contained 13 ± 3 bursts/dish. While lymphocyte membranes augment burst formation, human monocyte membranes suppress growth in a dose-related fashion ($P < 0.05$ for each).

It is possible that antimembrane IgG may differentiate between lymphocyte and monocyte-derived BPA or that the growth factor(s) may operate via different cell receptors. To further determine whether antimembrane IgG differentially recognizes lymphocyte- and monocyte-derived BPA, increasing amounts of IgG were added to supernatants prepared from LCM and MCM. Antibodies and antigen-antibody complexes were removed by incubation with an excess amount of Staphylococcal protein A and centrifugation. Absorbed supernatants were assayed for BPA in serum-free human marrow culture. Figure 5 shows that antimembrane IgG effectively removed BPA from LCM supernatant in a dose-related fashion. However, BPA in MCM supernatant was not removed, even when 200 μg antimembrane IgG/mL was used for adsorption. Together, these results suggest that lymphocyte- and monocyte-derived soluble BPA molecules may be antigenically distinct.

DISCUSSION

While cellular interactions within the marrow microenvironment are believed to be important in controlling the proliferation of hematopoietic cells,[18-20] little is known about

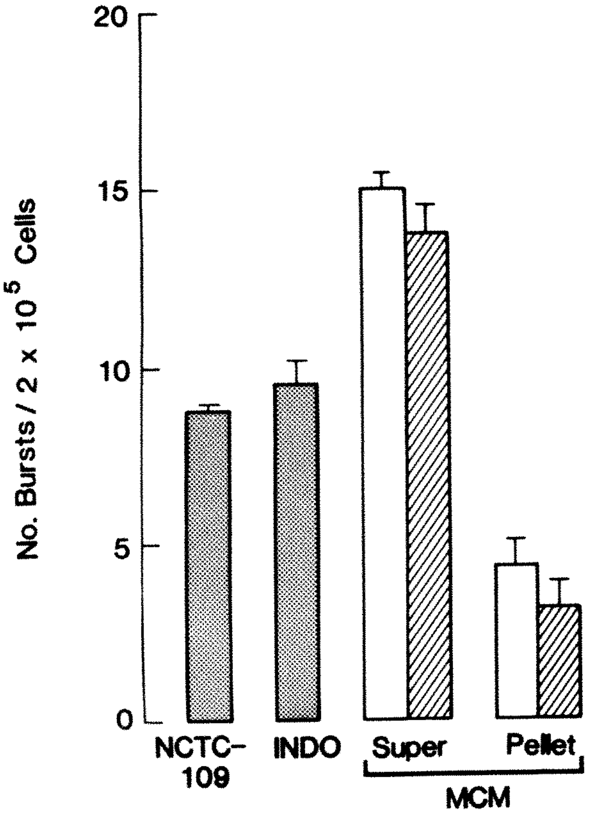

Fig 4. Effect of indomethacin on BPA expression. At the onset of 24-hr incubation at 37 °C, 10^{-6} mol/L indomethacin was added to a portion of adherent human monocyte-enriched circulating cells. Mean ± SEM burst formation is displayed for two studies in methylcellulose cultures containing 10% (v/v) NCTC-109, 10^{-6} mol/L of indomethacin added directly to culture (INDO), or MCM prepared from indomethacin exposed (hatched bars) and unexposed (open bars) cells. Similar results were observed in cultures containing LCM prepared from indomethacin-treated and untreated lymphocytes (data not shown).

how such communication occurs. Results of experiments in a number of tissue culture systems indicate that cell membrane-associated materials may be mitogenic for neighboring cells.[21,22] Studies in our laboratory have shown that erythroid BPA is likewise expressed by shed cell surface-derived vesicles and by plasma membranes of human lymphocytes. In this communication, we explore the capacity of human and murine monocytes to release surface-derived erythroid growth factors.

Our data indicate that in contrast to lymphocyte BPA, which is expressed by both soluble and membrane vesicle-containing CM fractions, only membrane vesicle-free CM supernatants from monocytes are capable of supporting BFU-E growth. Moreover, monocyte membranes and exfoliated membrane vesicles suppress burst proliferation, analogous to well-known contact inhibition of cell proliferation described in other culture systems.[23-25] This suppression does not appear to be mediated by prostaglandin release, since addition of indomethacin to cells during CM production does not reduce the inhibitory activity (see Fig 4). We cannot exclude the possibility that monocytes also produce membrane-associated BPA but that its expression is overwhelmed by concomitant release of a membrane-associated inhibitor. Experiments underway involving solubilization and isolation of lymphocyte membrane-derived BPA may also be applied to monocyte membranes in an effort to address this question.

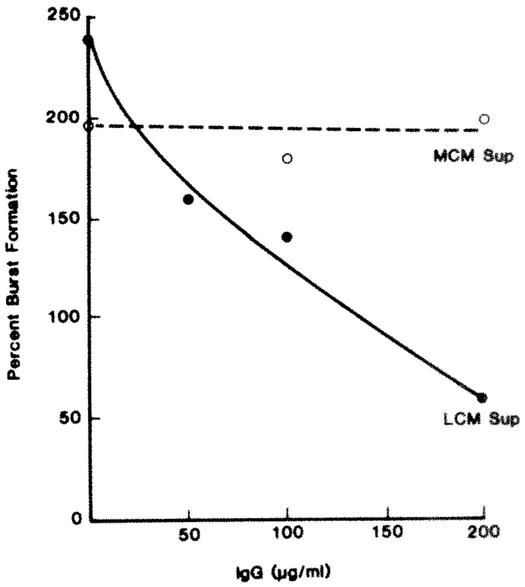

Fig 5. Effect of preadsorption of supernatants from LCM and MCM with IgG on BPA expression. The indicated amounts of IgG were incubated with 200 µL of supernatant for 16 hr, 4 °C, and then immunoprecipitated with Pansorbin. Absorbed supernatants (10%, v/v) (Sup) were assayed for BPA in serum-free cultures containing 2.0 IU/mL erythropoietin. Control cultures, containing CM supernatants that were sham-treated with Pansorbin in the absence of IgG, contained 12 to 14 bursts/dish. Note that IgG selectively removes BPA from supernatants prepared from LCM but not from MCM.

Our previous studies have shown that antilymphocyte membrane IgG is capable of neutralizing both soluble and vesicular lymphocyte-derived BPA in culture in a dose-related fashion.[12] Present experiments involving addition of various amounts of LCM or MCM supernatant to IgG-containing cultures suggest that soluble lymphocyte- and monocyte-derived BPA are antigenically distinct. That the antibodies involved are acting directly on a soluble factor and not on receptors of target cells in culture is demonstrated by our immunoadsorption experiments wherein IgG is removed prior to the addition of conditioned media to culture. Together, these results suggest that soluble BPA molecules released from monocytes are antigenically distinct from those derived from lymphocytes. The extent to which these two forms of the growth factor actually differ are now being addressed using monoclonal and polyclonal antibodies raised against partially purified lymphocyte BPA.

Since many potential interactions may occur among cells, growth factors, and essential tissue culture constituents,[26] it is possible that monocyte-derived mitogens operate at one or more levels to exert a positive or negative effect on BFU-E formation. Figure 6 depicts a hypothetical model of interactions between monocytes and other cell types present in unfractionated marrow cultures, the result of which may

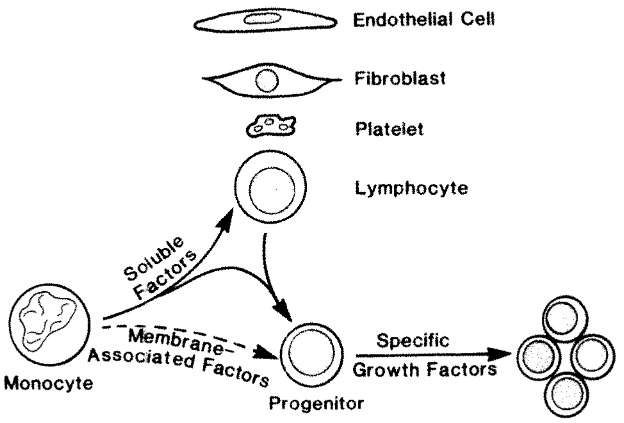

Fig 6. Hypothetical model of potential cellular interactions that may result in altered BFU-E proliferation. Solid arrows, positive regulatory effect; dashed arrow, negative regulatory effect.

alter erythroid progenitor cell proliferation in the presence of a specific differentiation factor such as erythropoietin. Evidence has accumulated to suggest that regulatory interactions may occur between hematopoietic progenitors and endothelial cells,[27,28] fibroblasts,[29-31] and platelets,[32] as well as with lymphocytes and monocytes.[7,8,33] Accordingly, it is possible that cytosolic or soluble membrane-derived components released from monocytes might augment the production of growth-promoting molecules from one or more of these cell types, or that they may act in concert with such molecules to initiate progenitor cell division. A similar level of activity has been proposed for the release of colony-stimulating activity from vascular endothelial cells[28] and fibroblasts,[31] and for the stimulatory action of platelet-derived growth factor on erythropoiesis in vitro.[34] Furthermore, Zuckerman and others have suggested that peripheral blood BFU-E proliferation is dependent on the presence of both monocytes and T lymphocytes, and that monocytes at high cell concentration exhibit inhibitory activity.[7-9] Our results are consistent with this dual monocyte effect and appear to have partially characterized the physical nature of the presumed inhibitor. In contrast to the action of soluble molecules, monocyte plasma membrane–associated suppressor activity might exert a direct effect on the BFU-E (see Fig 6).

In summary, our work supports the existence of multiple forms of a growth factor having erythroid burst-promoting activity. Moreover, it points to a potential dual role for monocytes in regulation of erythropoiesis wherein soluble monocyte products enhance and membrane-associated molecules suppress erythroid differentiation. The presence in these cells of two physically separable factors which exert opposing growth regulatory effects in vitro may provide an explanation for reports of dose-dependent effects of monocytes on human erythroid burst formation. Our findings indicate a unique opportunity to study the role of accessory cells in erythropoiesis at a subcellular level.

ACKNOWLEDGMENT

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REFERENCES

1. Aye MT: Erythroid colony formation in cultures of human marrow: Effect of leukocyte conditioned medium. J Cell Physiol 91:69, 1977

2. Iscove NN: Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned medium from lectin-stimulated mouse spleen cells, in Golde DW, Cline MJ, Metcalf D, Fox CF (eds): Hematopoietic Cell Differentiation (ICN-UCLA Symposium on Molecular and Cellular Biology, Vol 10). Orlando, Fla, Academic Press, 1978, pp 37–52

3. Nathan DG, Chess L, Hillman DG, Clarke B, Breard J, Merler E, Houseman DE: Human erythroid burst-forming unit: T cell requirement for proliferation in vitro. J Exp Med 147:324, 1978

4. Mangan KF, Desforges JF: The role of T lymphocytes and monocytes in the regulation of human erythropoietic peripheral blood burst forming units. Exp Hematol 8:717, 1980

5. Gordon LI, Miller WJ, Branda RF, Zanjani ED, Jacob HS: Regulation of erythroid colony formation by bone marrow macrophages. Blood 55:1047, 1980

6. Dainiak N, Cohen CM: Surface membrane vesicles from mononuclear cells stimulate erythroid stem cells to proliferate in culture. Blood 60:583, 1982

7. Zuckerman KS: Stimulation of BFU-E by products of human monocytes and lymphocytes. Exp Hematol 8:924, 1980

8. Grilli G, Carbonell F: Effect of blood derived monocytes on the promotion of in vitro erythropoietic colony growth in human bone marrow cultures. Scand J Haematol 29:345, 1982

9. Hanada T, Nagasawa T, Abe T: Dual effect of monocyte-conditioned medium on in vitro hematopoiesis. Exp Hematol 10:561, 1982

10. Perper RJ, Zee TW, Mickelson MM: Purification of lymphocytes and platelets by density gradient centrifugation. J Lab Clin Med 72:5, 1978

11. Jett M, Steed TM, Jamieson GA: Isolation and characterization of plasma membranes and intact nuclei from lymphoid cells. J Biol Chem 252:2134, 1977

12. Dainiak N, Feldman L, Cohen CM: Neutralization of erythroid burst promoting activity in vitro with antimembrane antibodies. Blood 65:877, 1985

13. Dainiak N, Kreczko S, Cohen AM, Pannell R, Lawler J: Primary human marrow cultures for erythroid bursts in a serum-substituted system. Exp Hematol 13:1073, 1985

14. Goldstein A: Biostatistics; An Introductory Text. New York, Macmillan, 1964, p 62

15. Dukes PP, Shore NA, Hammond D, Ortega JA, Datta MC: Enhancement of erythropoiesis by prostaglandins. J Lab Clin Med 82:704, 1973

16. Rossi GB, Migliaccio AR, Migliaccio G, Lettieri F, DiRosea M, Mastroberardino G, Peschle C: In Vitro interactions of PGE and cAMP with murine and human erythroid precursors. Blood 56:74, 1980

17. Motomura S, Dexter TM: The effect of prostaglandin E on hemopoiesis in longterm bone marrow cultures. Exp Hematol 8:298, 1980

18. Cline MJ, Golde DW: Cellular interactions in haematopoiesis. Nature 277:177, 1979

19. Zanjani ED, Kaplan ME: Cell-cell interaction in erythropoiesis. Prog Hematol 11:173, 1979

20. Tavassoli M, Friedenstein A: Hemopoietic stromal microenvironment. Am J Hematol 15:195, 1983

21. Salzer JL, Bunge RP, Glaser L: Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. J Cell Biol 84:767, 1980

22. Lodish HF, Chisholm L, Barklis E, Cohen SM: Dictyosteliium differentiation: Sequential induction of cell type specific mRNAs is mediated by developmentally regulated cell surface proteins. J Cell Biochem 8A:233, 1984 (Suppl)

23. Vale RD, Peterson SW, Mativek NV, Fox CF: Purified plasma membranes inhibit polypeptide growth factor-induced DNA synthesis in subconfluent 3T3 cells. J Cell Biol 98:1129, 1984

24. Stallcup KC, Dawson A, Mescher MF: Growth-inhibitory activity of lymphoid cell plasma membranes. I. Inhibition of lymphocyte and lymphoid tumor cell growth. J Cell Biol 99:1221, 1984

25. Healy CT, Kapp JA, Webb DR: Purification and biochemical analysis of antigen-specific suppressor factors obtained from the supernatant, membrane, or cytosol of a T cell hybridoma. J Immunol 131:2843, 1983

26. Dainiak N: Role of defined and undefined serum additives to hematopoietic stem cell culture, in Cronkite EP, Dainiak N, McCaffrey R, Palek J, Quesenberry P (eds): Hematopoietic Stem Cell Physiology. New York, Alan R. Liss, 1985, pp 59–76

27. Ascensao JL, Vercellotti GM, Jacob HS, Zanjani ED: Role of endothelial cells in human hematopoiesis: modulation of mixed colony growth in vitro. Blood 63:553, 1984

28. Bagby GC, McCall E, Bergstrom KA, Burger D: A monokine regulates colony-stimulating activity production by vascular endothelial cells. Blood 62:663, 1983

29. Ghio R, Bianchi G, Lowenberg B, Dicke KA, Ajmoar F: Effects of fibroblasts on the growth of erythroid progenitor cells in vitro. Exp Hematol 5:341, 1977

30. Gordon MY, King JA, Gordon-Smith EC: Bone marrow fibroblasts, fat cells and colony-stimulating activity. Brit J Haematol 46:151, 1980

31. Bagby GD, McCall E, Layman DL: Regulation of colony-stimulating activity production. Interactions of fibroblasts, mononuclear phagocytes, and lactoferrin. J Clin Invest 71:340, 1983

32. Dainiak N, Davies G, Kalmanti M, Lawler J, Kulkarni V: Platelet-derived growth factor promotes proliferation of erythropoietic progenitor cells in vitro. J Clin Invest 71:1206, 1983

33. Gordon MY, Gordon-Smith EC: Annotation: Lymphocytes and haemopoiesis. Brit J Haematol 47:163, 1981

34. Delwiche F, Raines E, Powell J, Ross R, Adamson J: Platelet-derived growth factor enhances in vitro erythropoiesis via stimulation of mesenchymal cells. J Clin Invest 76:137, 1985

Fibrinopeptide A and the Phosphate Content of Fibrinogen in Venous Thromboembolism and Disseminated Intravascular Coagulation

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W.G. van Aken, and J.A. van Mourik

Concentrations of plasma fibrinopeptide A (FPA) were measured by radioimmunoassay in 50 patients with venous thromboembolism or disseminated intravascular coagulation or both. A consistent discrepancy was observed in values obtained with two anti-FPA antisera. Analysis of extracts from plasma of these patients by high-performance liquid chromatography (HPLC) revealed the presence of a phosphorylated and an unphosphorylated form of the A peptide. Differences in concentrations of FPA measured with the two antisera could be accounted for by their different reactivity with phosphorylated FPA (FPA-P). The differences were abolished by treatment with alkaline phosphatase. A good correlation was observed between the FPA-P content of free A-peptide material and of fibrinogen in plasma as determined by HPLC ($r = .88$, $P < .001$, n = 11). In patients with elevated FPA levels, the mean FPA-P content of fibrinogen was significantly higher ($P < .002$, n = 13) than in patients with normal FPA levels (n = 8) and in healthy controls (n = 14). Phosphorus in fibrinogen did not correlate with fibrinogen degradation products or fibrinogen levels and became normal on adequate anticoagulation. Therefore, blood-clotting activation may lead to a high phosphate content of fibrinogen and of free FPA in plasma.

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THROMBIN CATALYZES the removal of two pairs of small peptides from fibrinogen, namely fibrinopeptides A and B. The presence of these peptides in samples of clinical blood, therefore, may reflect thrombin activity in vivo. Nossel et al[1] introduced a radioimmunoassay of fibrinopeptide A (FPA), and they obtained promising results in this respect. Elevated levels were found in disorders assumed to be associated with intravascular formation of thrombin.[2-7] In studies on the effect of thrombin on human fibrinogen in vitro, Blombäck et al[8] identified three forms of the A peptide, which indicated a potential heterogeneity of peptide material released from fibrinogen. The major product, the $A\alpha 1$-16 fragment of the α-chain of fibrinogen was considered to be the original, native fibrinopeptide A. About 30% of this peptide contained a phosphate group, covalently attached to the third amino acid, serine, and was designated FPA-P. A small portion of the A-peptide material lacked the amino-terminal amino acid (alanine) and was named Y-peptide. No information is so far available as to the question whether these various forms of the A peptide circulate in vivo.

We have produced a series of rabbit-anti-FPA sera that enabled us to perform a more detailed analysis of the immunoreactive material in samples of clinical blood. High-performance liquid chromatography (HPLC) of plasma extracts, combined with radioimmunoassays with two antisera having different specificities, showed that, in certain clinical conditions, FPA may circulate predominantly in its phosphorylated form. The latter situation appears to be invariably associated with an increased phosphorylation of plasma fibrinogen as well.

MATERIALS AND METHODS

Anti-FPA antisera. Antiserum R 6216 has been described previously.[3] R44656 was obtained by procedures described by Nossel et al,[1,2] except that rabbits were immunized with synthetic (Serva, Heidelberg, FRG) instead of native FPA coupled to human serum albumin (Serva; 5× crystallized) by the carbodiimide method.[9] Cross-reactivity of these sera with purified human fibrinogen, prepared as described[10] (a gift from Drs. F. Haverkate and W. Nieuwenhuizen, Gaubius Institute—TNO, Leiden, The Netherlands), was determined according to Canfield et al.[11] When the immunoreactivity with synthetic FPA (Schwarz Mann, Orangeburg, SC, unless stated otherwise) was defined as 100%, fibrinogen showed a cross-reactivity of 33% with R 6216 and about 0.2% with R 44656.

Collection and processing of blood. After clean venipuncture, 4.5 mL of venous blood was collected in siliconized Vacutainer tubes, containing 0.5 mL of an anticoagulant mixture of 500 units of heparin (Kabi Vitrum, Stockholm), 500 U of Trasylol (Bayer, Leverkussen, FRG), and 19 mg trisodium citrate 0.2 aq. The tubes were shaken, placed in melting ice, and centrifuged for ten minutes at 4 °C and 2,000 g, the whole procedure being completed within 30 minutes after venipuncture. Plasma proteins were immediately precipitated by mixing (in duplicate) 0.8 mL of plasma with 0.8 mL of 40% ice-cold PEG (PEG 6000; Merck, Darmstadt, FRG) in phosphate-buffered saline (0.14 mol/L NaCl, 0.010 mol/L Na_2HPO_4, pH 7.4). After incubation for ten to 20 minutes in an ice bath, these mixtures were centrifuged at 4 °C for 30 minutes at 2,000 g. The supernatants were heated for two minutes in a boiling water bath. Thereafter, tubes were centrifuged at room temperature for ten minutes at 2,000 g; then 0.6-mL aliquots of supernatants were stored at −30 °C or used directly for assay. When synthetic FPA was added to five citrated plasmas in six concentrations, ranging from 6.25 to 200 ng/mL, the mean recovery was 103% (94% to 116%) as determined by radioimmunoassay with R 6216.

Radioimmunoassay of FPA. The immunoassay was performed as described,[3] except that PEG precipitation instead of dialysis was used to separate low-molecular-weight FPA-immunoreactive material from fibrinogen. A mixture of PEG, citrate, heparin, and Trasylol in assay buffer (0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.5, 0.25% ovalbumin, Serva) in concentrations equivalent to 1:1 diluted sample supernatant was used to produce the standard curve. Values

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in specimens from 70 healthy controls (aged 18 to 68 years) ranged from undetectable to 2 ng/mL. The value 2 ng/mL was therefore chosen as the upper limit of the normal range, with a 95% to 99% confidence interval.

Material from patients. Fifty patients with clinical signs of venous thromboembolism or disseminated intravascular coagulation (DIC) or both were studied. The presence of deep venous thrombosis was established by venography in 24 patients and suggested by abnormal impedance plethysmography in three. One patient (No. 3, Table 1) had a thrombophlebitis migrans (no venography performed). In ten patients, the appearance of multiple segmental or lobar perfusion defects in two or more views on a technically adequate perfusion scan, in the presence of a normal chest x-ray, suggested pulmonary embolism. A Xenon-133 ventilation scan, performed in two of these patients, supported the diagnosis of pulmonary embolism.

In 12 patients, a diagnosis of diffuse intravascular coagulation was made. All but two of these patients had elevated FDP/fdps.[12] The ethanol-gelation test[13] was positive in six. In addition, eight patients showed a low platelet count, and a low fibrinogen concentration was present in four of them. DIC was attributed to disseminated neoplasia in nine, and in the other three, to severe acute hemolytic anemia of unknown etiology, chronic myeloid leukemia, and an autoimmune disorder (No. 9, Table 1). Blood was only obtained after informed consent from the patient, and our study was approved by a local ethical committee.

Purification of fibrinopeptides. One gram of fibrinogen (Kabi; grade L) was dissolved in 100 mL of distilled water and allowed to

Table 1. Two Groups of Patients in Whom the FPA-P Content of Fibrinogen was Assessed

Patient	Age	Sex	Diagnosis	FDP/fdp (μg/mL)	Platelets (10^9/L)	Serum Alkaline Phosphatase (U/L)	FPA (ng/mL)*		Fibrinogen	
							R44656	R6216	(g/L)	FPA-P/Total FPA (%)
With venous thromboembolism or DIC										
1. A.S.	41	F	Metastasized ovarian carcinoma, Denver shunt, DIC	20.0	64	89	23.4	10.2	4.5	62
2. G.K.	74	F	Metastasized rectal carcinoma, DIC	320.0	74	—	21.1	5.7	0.9	72
3. J.B.	64	F	Metastasized endometrical carcinoma, thrombophlebitis migrans	<5.0	225	84	8.8	7.0	3.9	51
4. A.O.	63	M	Metastasized pancreatic carcinoma, thrombophlebitis migrans, DIC	320.0	235	980	11.8	8.8	3.9	35
5. J.V.	33	M	Deep venous thrombosis, alcohol abuse, no concurrent disorder	≤40.0	704	—	11.0	7.9	6.2	41
6. M.R.	68	F	Metastasized ovarian carcinoma, diabetes mellitus, pulmonary embolism	≤1.25	210	123	22.0	9.3	4.9	66
7. C.Z.	84	M	Metastasized rectal carcinoma, pulmonary embolism	≤1.25	120	258	8.8	6.1	4.0	48
8. H.V.	38	F	Metastasized ovarian carcinoma, pulmonary embolism	≤1.25	330	265	5.1	2.1	4.0	61
9. J.W.	27	F	Autoimmune disorder (juvenile rheumatoid arthritis, SLE?), DIC	≤1.25	19	345	4.5	1.7	1.1	58
10. M.P.	59	M	Deep venous thrombosis, no concurrent disorder	≤1.25	174	44	4.7	3.7	3.5	29
11. F.R.	68	M	Non-Hodgkin lymphoma, thrombophlebitis migrans	10.0	155	104	3.6	2.1	2.5	50
12. H.W.	73	M	Metastasized prostatic carcinoma, DIC	<2.5	33	218	27.2	4.8	2.2	67
13. H.D.	64	F	Metastasized ovarian carcinoma, pulmonary embolism	<2.5	303	175	6.8	2.6	5.1	55
Without venous thromboembolism or DIC										
14. A.H.	71	M	Metastasized nasopharyngeal carcinoma	<2.5	246	—	1.6	0.8	3.9	28
15. M.S.	41	F	Metastasized breast carcinoma	<2.5	214	63	1.6	0.1	—	26
16. P.O.	71	F	Metastasized carcinoid tumor	<2.5	234	220	1.2	0.6	3.9	35
17. R.B.	30	M	Metastasized melanoma	≤1.25	185	51	0.0	0.0	1.5	19
18. C.L.	44	F	Metastasized breast carcinoma	≤1.25	295	349	1.9	0.8	4.1	22
19. A.K.	27	F	Acute myelomonocytic leukemia	≤1.25	70	71	1.6	1.0	2.7	32
20. M.H.	59	F	Metastasized breast carcinoma	≤1.25	228	66	0.0	1.5	3.3	38
21. J.D.	70	F	Vulva carcinoma	<5.0	350	100	1.6	0.4	6.7	39
Normal values				<10	150–300	35–110	<2	<2	2–4	16–42†

FPA levels and the content of FPA-P in fibrinogen in patients of group I differ significantly ($P < .002$, Wilcoxon-White, two-sample ranks test) from those of group II. Differences in platelet counts, fibrinogen concentrations, FDP levels, and serum concentrations of alkaline phosphatase are not significant ($P > .1$). FPA levels from the two groups together, as determined with R 44656, correlate significantly with the content of phosphopeptide in fibrinogen ($y = 1.4 \times + 32.8$, $n = 21$, $r = .76$, $P < .001$). The correlation found by using the FPA concentrations measured with R 6216 was less ($y = 2.5 \times + 35.2$, $n = 21$, $r = .53$, $P = .05$). No correlation was found by either linear regression or ranks analysis between the content of FPA-P in fibrinogen and FDP levels, platelet counts, or concentrations of fibrinogen or serum alkaline phosphatase.

*Values denote concentrations of free A peptide in plasma as determined by radioimmunoassay with either R 44656 or R 6216.

†Mean ± 2 SD, as determined in healthy controls ($n = 14$).

clot for two to 24 hours at 37 °C by adding 1 unit of human thrombin per milliliter (500 U/mL, Sigma, St Louis). The fibrin supernatant was mixed with an equal volume of ice-cold ethanol (Merck) and incubated for 20 minutes in an ice bath. Supernatants were heated for two minutes at 100 °C, mixed once more with an equal volume of ethanol, and recentrifuged (30 minutes, 15,000 rpm, 4 °C). This supernatant (about 400 mL) was evaporated to dryness by rotary film evaporation at 60 °C and taken up in 4 mL 1% acetic acid. This volume was reduced to about 1 mL by blowing a stream of nitrogen over the sample at 40 °C. The material was then spun for four minutes at 8,000 g. Next, 0.4 mL of the supernatant was chromatographed on a Biogel-P6 column, 60 × 0.9 cm (Bio-Rad, Richmond, Calif), equilibrated with 1% acetic acid (vol/vol), 1 mmol/L NaH_2PO_4, pH 2.4. Immunoreactive material eluting from this column was lyophilized and dissolved in 10% (vol/vol) acetonitrile (Rathburn Chemicals, Edinburgh, Scotland; HPLC, grade S), 0.1% (vol/vol) phosphoric acid (Merck), pH 2.1.

Of this eluate, 75 to 100 μL (about 4 mg of A-peptide immunoreactivity per milliliter) was chromatographed on an octadecylsilica-C18 column (for conditions, see later). Alkaline phosphatase treatment of concentrated fibrin clot supernatant was performed as described for patients' samples (see later).

Amino acid analysis. After hydrolysis in 6 mol/L HCl in vacuo for 24 hours at 110 °C, fractions were analyzed by standard techniques on a Chromakon-500 amino acid analyzer (Kontron, Zurich).[14]

HPLC analysis of plasma-FPA immunoreactivity. Blood used for HPLC analysis was collected and centrifuged as described. Plasma was mixed with an equal volume of ice-cold ethanol and, after a 20-minute incubation period in an ice bath, spun for 30 minutes at 2,000 g at 4 °C. Supernatants were decanted, incubated for another five to ten minutes in an ice bath, and recentrifuged. Supernatants were then heated for two minutes in a boiling water bath, centrifuged for ten minutes at 2,000 g at room temperature, then stored in plastic tubes at −70 °C until further analysis. Disposable 6-mL octadecylsilica-(C18) columns (J.T. Baker Chemical Co, Philipsburg, NJ) were washed with 6 mL methanol and 18 mL of 0.1% phosphoric acid before the addition of samples. A single ethanol supernatant, usually 5 mL, was diluted tenfold in 0.1% phosphoric acid, pH 2.1, and applied on this column. After several washes with 0.1% phosphoric acid, peptide material was eluted with (5 × 0.5 mL) 50% acetonitrile in 0.1% phosphoric acid. The eluate was concentrated, under a stream of nitrogen at 40 °C, to 150 to 200 μL. Thereafter, samples were spun for two minutes at 8,000 g, and 0.1 mL supernatant was injected for HPLC analysis.

To test the effect of alkaline phosphatase treatment on the FPA immunoreactivity in these supernatants, duplicate samples were diluted in 5 mL 0.1% phosphoric acid and passed once more through a C18 column. Columns were washed with 0.1% phosphoric acid and then with distilled water, followed by elution of peptide material with 20% acetonitrile in 0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.0. Eluted material was pooled and concentrated under a stream of nitrogen to about 2 mL. Aliquots of 800 μL were then mixed with 2.2 mL of 0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.5. The mixture was incubated for two hours at 37 °C with or without 10 units calf intestine alkaline phosphatase per milliliter (Boehringer, Mannheim, FRG; 1,500 U/0.74 mg/0.146 mL). The reaction was stopped by mixing these 3-mL aliquots with 30 mL 0.1% phosphoric acid. To remove the enzyme, samples were again passed through a C18 column, equilibrated with 0.1% phosphoric acid; peptides were eluted with 50% acetonitrile in 0.1% phosphoric acid.

Reversed-phase HPLC was performed on a μ-Bondapack C18 column (0.39 × 30.0 cm) from Millipore Waters (Milford, Mass.), by using a Perkin-Elmer series 2 liquid chromatograph, equipped with a Rheodyne injector (0.1-mL loop). Gradient elution was achieved with a mobile phase consisting of acetonitrile and 0.1% phosphoric acid, pH 2.1. The concentration of acetonitrile was initially 10% and was programmed to increase ten minutes after application of the sample at the rate of 1.25% per minute. Chromatography was conducted at room temperature with a flow rate of 2 mL/min. Fractions of 0.6 mL were collected and then tested for FPA immunoreactivity. Tubes for the standard curves contained 0.1-mL aliquots of 15% acetonitrile in 0.1% phosphoric acid, 0.2 mL assay buffer containing various amounts of synthetic FPA, 0.1 mL tracer, and 0.1 mL diluted antiserum. Assay tubes for "fraction testing" contained 0.1-mL aliquots (10% to 30% acetonitrile in 0.1% phosphoric acid), 0.2 mL assay buffer, 0.1 mL tracer, and 0.1 mL diluted antiserum. Standard curves obtained with samples containing acetonitrile were identical with those obtained with 0.1-mL aliquots of diluted PEG (cf. standard radioimmunoassays) in a total assay volume of 0.5 mL.

Extent of phosphorylation of fibrinogen. Blood (containing 13 mmol/L trisodium citrate), collected on ice, was centrifuged within 30 minutes of venipuncture. Supernatant plasma (ten minutes, 2,000 g, 4 °C) was rendered platelet-poor (two minutes, 8,000 g, 4 °C) and stored in plastic tubes at −70 °C. To determine the fibrinopeptide A-P content of plasma fibrinogen by HPLC analysis, by using an ammonium acetate buffer,[15] in combination with radioimmunoassays with R 44656, plasma samples were rapidly thawed at 37 °C and incubated for 30 minutes at 37 °C with 20 units of human thrombin per milliliter (Sigma; 3,000 U/mg). Clot supernatants were mixed with β-glycerophosphate (Sigma) 100 mmol/L fc and precipitated with ethanol as described. Supernatant (100 μL) was added to 5 mL 0.1% phosphoric acid, pH 2.1, and analyzed by HPLC as described. FPA release from fibrinogen by thrombin was complete as determined by comparing the theoretical concentrations of A peptide with the measured concentrations. Recovery of FPA from disposable extraction columns and HPLC was >98%. The mean percentage of fibrinogen, phosphorylated at position $A\alpha$ Ser3, obtained from total plasma fibrinogen by this procedure, in healthy controls (n = 14, aged 18 to 55 years) was 29% (19% to 42%), in dogs (n = 2, four determinations) 68% (60% to 72%), and in full-term newborn infants (n = 3) 58% (54% to 65%).

Statistical methods. FPA values were not normally distributed; therefore, Spearman's rank test was used to determine the correlation between values measured with the two anti-FPA sera (Fig 1). The significance of the differences between values measured with the two antisera was tested by Wilcoxon's signed rank test (Fig 1). Comparisons between patients with venous thromboembolism and control patients or normal volunteers were made with the Wilcoxon-White two-sample ranks test (Table 1). The possibility of a correlation between the phosphopeptide content of fibrinogen and the FPA levels, the fibrinogen concentration, the platelet count, the FDPs, and the alkaline phosphatase levels was tested by both linear regression and rank analysis, the latter by using Spearman's rank test as well as the Kendall rank correlation coefficient test (Table 1). Comparisons between results obtained before and during anticoagulation were analyzed by using Student's t test for paired data.

RESULTS

FPA in plasma from patients with venous thromboembolism or DIC or both. When antiserum R 44656 was used in the radioimmunoassay, elevated concentrations of FPA were observed in plasma from 40 out of 50 patients with venous thromboembolism or DIC or both. With antiserum R 6216, FPA concentrations were increased in 35 of these patients. The values found with the two antisera differed significantly ($P < .0005$). R 44656 reacted apparently almost invariably

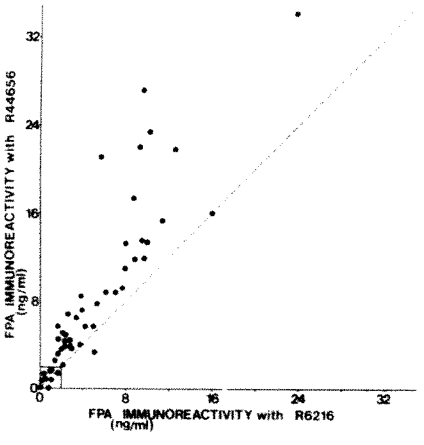

Fig 1. **Plasma FPA levels as measured with two anti-FPA sera (R 44656 and R 6216) in 70 normal individuals depicted within a square, 50 patients with venous thromboembolism or DIC or both (●). The broken line indicates the hypothetical ideal relationship between the results obtained with the two sera. The observed correlation for the thrombosis patients was: $r_s = .91$, $P < .001$ (Spearman's rank test). Differences between values measured with the two sera were highly significant ($P < .0005$ Wilcoxon's signed rank test).**

with more material in PEG supernatants from plasma than did R 6216 (Fig 1). Administration of heparin resulted in all patients with venous thromboembolism, in which the effect of this drug was evaluated, for both antisera in a prompt decline of plasma-FPA immunoreactivity (Fig 2).

HPLC of fibrinopeptides. In view of the dissimilar reactivity of two anti-FPA sera with the immunoreactive material in clinical blood samples, it was thought that plasma-FPA immunoreactivity was heterogeneous. To test this hypothesis, native fibrinopeptides and immunoreactive material in plasma were analyzed by HPLC. Figure 3A shows a chromatogram of a fibrin clot supernatant, obtained by allowing purified human fibrinogen to clot with thrombin for two hours at 37 °C. Four major peaks were eluted with the acetonitrile gradient. Peaks I and II had an identical composition, corresponding to that of FPA, whereas the amino acid composition of peaks III and IV corresponded to fibrinopeptide B and its degradation product Bβ 1-13, respectively. These results were in agreement with those of Koehn et al,[16] who identified peak I as the unphosphorylated and peak II as the phosphorylated forms of FPA. Peak I exhibited similar FPA immunoreactivity with our two sera. Peak II, in contrast, reacted a hundredfold less with R 6216 as compared with R 44656 (Fig 3A and B). Incubation of peak II with alkaline phosphatase had no effect on the reactivity of R 44656, but abolished the difference in reactivity between the two sera. Treatment of concentrated fibrin clot supernatant with alkaline phosphatase resulted in a complete disappearance of peak II and a corresponding relative increase in peak I, whereas peaks III and IV were unchanged (Fig 4). These results suggested that R 6216 was directed to antigenic sites of the peptide that became exposed on removal of the serine-bound phosphate. This finding provided a potential explanation for the dissimilar reactivity of the two sera with material from patients.

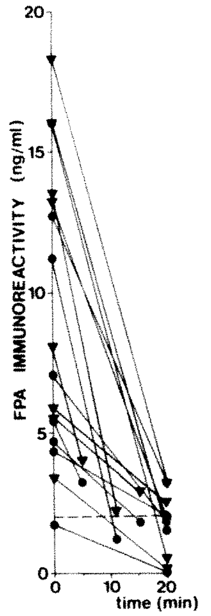

Fig 2. **Effect of a bolus injection of heparin (5,000 to 7,500 units) on plasma-FPA immunoreactivity in eight patients with venous thromboembolism. The horizontal broken line denotes the upper limit of the normal range. Time between blood sampling is indicated on the abscissa: O, values before heparin; ▼, data with R 44656; ●, data with R 6216. Values measured with R 44656 or R 6216 declined after administration of heparin, suggesting that material detected with the two sera is a result of thrombin activity.**

HPLC of plasma-FPA immunoreactivity from patients with venous thromboembolism or DIC or both. To investigate whether FPA immunoreactivity in plasma from patients consisted of a mixture of phosphorylated and unphosphorylated FPA, plasma samples were analyzed by HPLC. For practical reasons, ethanol instead of PEG supernatants were used. This was legitimate because experiments indicated that ethanol and PEG supernatants yielded identical HPLC patterns (not shown).

Figure 5A shows a chromatogram of a plasma extract from a patient with DIC. Two major peaks of FPA immunoreactivity were eluted from the column; the first was recognized equally well by both sera. The second peak reacted (almost) exclusively with antiserum R 44656 and disappeared completely if samples were pretreated with alkaline phosphatase (Fig 5B). HPLC-purified native fibrinopeptide A as well as synthetic FPA were added as internal standards to patients' samples and were eluted at exactly the same position as the first peak, whereas FPA-P purified by HPLC behaved identically to peak II. FPA immunoreactivity containing fractions from Fig 5A were pooled, concentrated, and rerun with an ammonium acetate buffer as described by Kehl et al.[15] This experiment provided an additional argument that peak II represented FPA-P and peak I, FPA because, as expected, the order of elution of these compounds reversed under these conditions (Fig 5C).

HPLC analysis of plasma-FPA immunoreactivity was performed in 14 patients with venous thromboembolism. A phosphorylated form and an unphosphorylated form of the A

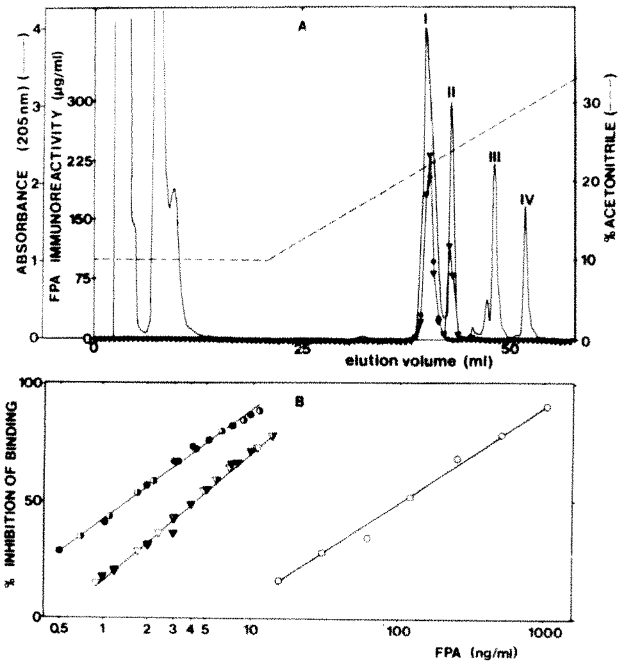

Fig 3. (A) Analysis of a fibrin clot supernatant by HPLC. Seventy-five microliters of concentrated clot supernatant, containing about 400 µg of FPA immunoreactivity, was injected and then eluted with an acetonitrile gradient as indicated. Fractions of 0.6 mL were collected for radioimmunoassay and amino acid analysis. I, FPA; II, FPA-P; III, FPB, IV, Bβ 1-13, ▼—▼, R 44656, ●—●, R 6216. (B) Reactivity with synthetic FPA, native FPA, and phosphorylated FPA of two anti-FPA sera. We tested 100-µL volumes, containing various concentrations of fibrinopeptide A or AP, determined by amino acid analysis. On the ordinate: the observed inhibition of binding of ^{125}I-Tyr FPA. Triangles depict values with antiserum R 44656; circles, values with R 6216. ▼, ●, synthetic FPA; ▼, ◑, native FPA (peak I, Fig 3A); ▽, ○, native FPA-P (peak II, Fig 3A).

peptide were always observed. The ratio between these two forms varied considerably from one individual to another. As no dephosphorylation of FPA-P added immediately after collection of blood was observed and addition of even high concentrations of phosphatase inhibitors, such as β-glycerophosphate (up to 0.1 mol/L), either to the anticoagulant mixture or to plasma before precipitation with ethanol, had no effect on the FPA-P–FPA ratio (data not shown), the presently observed variation appears to reflect the situation in vivo.

Phosphate content of fibrinogen in patients with venous thromboembolism or DIC or both. In several patients with high FPA levels, well over 30% of the A peptide was in its phosphorylated form (cf. Fig 5). If this was a reflection of a high FPA-P content of fibrinogen, a high phosphorus content in fibrinogen might be related to coagulation activation in vivo. Therefore, the FPA-P content in fibrinogen was determined by HPLC in patients with venous thromboembolism or DIC or both (n = 13), selected from the 50 patients depicted in Fig 1 on the basis of elevated FPA levels, as well as in control patients (n = 8) without venous thromboembolism or DIC and without elevated FPA levels (Table 1). The mean phosphorus in fibrinogen from patients with venous throm-

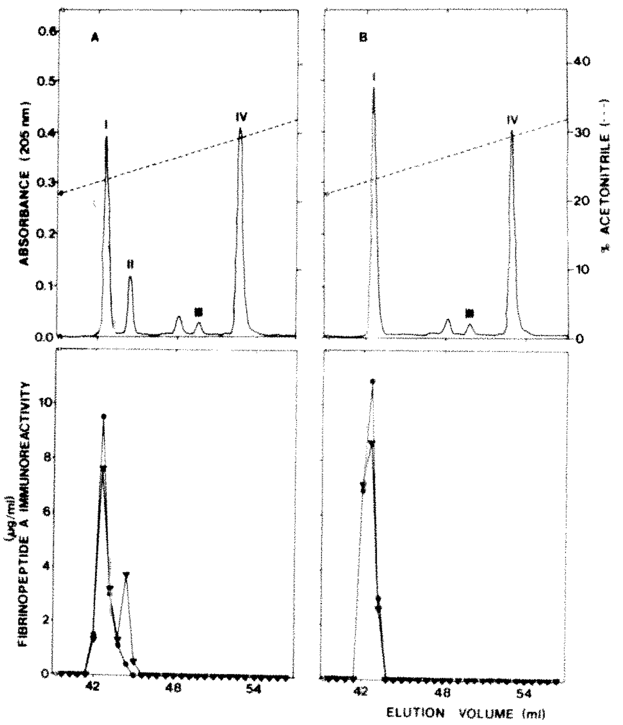

Fig 4. HPLC analysis of fibrin clot supernatant before and after treatment with alkaline phosphatase. Fibrinogen was clotted for 24 hours with 1 unit of thrombin per milliliter. The supernatant was passed through a disposable C18 column. The eluate, containing 0.1 mg of A-peptide immunoreactivity per milliliter, was incubated for two hours at 37 °C in 0.1 mol/L NaCl, 0.05 Tris, pH 8.5, in the absence (A) or presence (B) of alkaline phosphatase (10 U/mL). HPLC conditions were identical with those in Fig 3. One hundred microliters (~13 µg A-peptide immunoreactivity) were injected. Absorbance was monitored at 205 nm and 0.6-mL fractions were tested with R 6216 (●—●) and R 44656 (▼—▼).

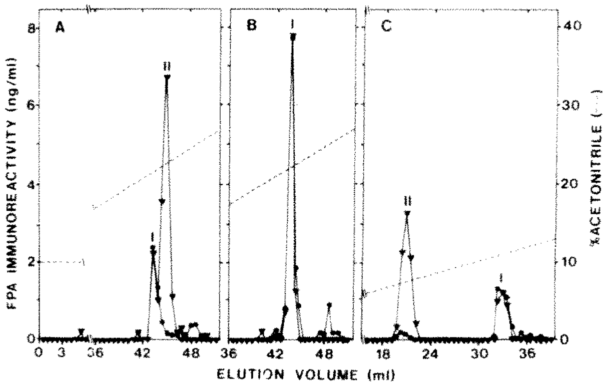

Fig 5. (A) HPLC analysis of plasma-FPA immunoreactivity from a 76-year-old patient, V., with a metastasized small cell lung carcinoma and signs of DIC. FDP/fdp, 80 µg/mL; fibrinogen, 1.5 mg/mL; platelets, 58 × 10^9/L; factor II, 54%; factor V, 90%; factor VII, 68%; and factor X, 70%. FPA immunoreactivity (as measured in PEG supernatant): R 44656, 8.7 ng/mL; R 6216, 3.3 ng/mL. (B) Effect of pretreatment with alkaline phosphatase. Control incubation without the enzyme had no effect (not shown). (C) FPA immunoreactivity-containing fractions from A and the control were rerun in an ammonium acetate buffer.[15] FPA immunoreactivity was measured in each fraction with antisera R 44656 (▼—▼) and R 6216 (●—●).

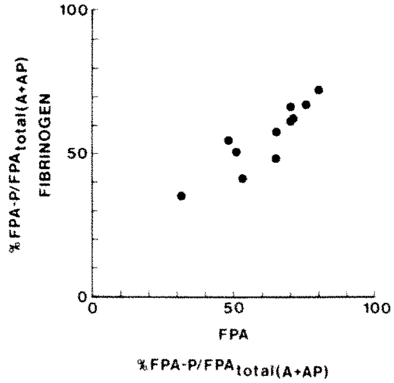

Fig 6. Comparison between the FPA-P content of fibrinogen and of the free A-peptide population in plasma from patients with venous thromboembolism and elevated FPA levels (Table 1, Group I). Linear regression analysis showed a correlation coefficient of +0.88 ($P < .001$) and the following equation: y = 0.7 × +12.2, with a standard deviation of the slope of 0.125 and of the intercept of 7.9. The non-zero intercept is not significantly different from zero.

boembolism and elevated FPA levels (group I) was significantly higher ($P < .002$) than in control patients (group II) and healthy individuals. The FPA-P content in fibrinogen correlated significantly ($P < .001$) with FPA levels, but not with the level of fibrin or fibrinogen degradation products, the plasma fibrinogen concentration, the platelet count, or the serum alkaline phosphatase concentration. In 11 patients from group I, the FPA-P total FPA ratio was analyzed in fibrinogen as well as in the population of free FPA present in the circulation. An excellent correlation ($r = .88$, $P < .001$) was observed between the two analyses (Fig 6).

Differences in FPA levels measured in group I with R 44656 and R 6216 in plasma PEG supernatants in general reflected the extent of phosphorylation of fibrinogen. In several patients, however, the FPA-P content in fibrinogen, as calculated from the data on free FPA in plasma PEG supernatants, was lower than actually observed by HPLC analysis. Differences in FPA concentrations determined with the two antisera in plasma from control patients (group II) were no reflection of the FPA-P content in fibrinogen, which is not surprising because values below 2 ng/mL plasma could not be measured accurately enough.

Nine patients from group I received anticoagulant treatment. Its effect on the FPA-P content in fibrinogen was studied in six. Adequate anticoagulation (ie, a doubling in the activated partial thromboplastin time (APTT), or thrombotest values <10%) was achieved in four (No. 5, 6, 8, 13). The FPA-P content in fibrinogen in these four patients was significantly lower ($P < .0025$), when FPA concentrations had become normal on therapy with heparin and oral anticoagulants, than before (mean before 56% [41% to 66%], during 32% [20% to 41%]). On-line studies were performed in three of these patients and are shown in Fig 7. From the two remaining inadequately anticoagulated patients, receiving only low-dose heparin (No. 2) or only oral anticoagulants (No. 3), one sample was obtained during therapy (nine and 14 days after initiation of therapy) showing no normalization of the phosphopeptide content in fibrinogen (before 72% and 51%; during 72% and 48%) or of the FPA level (determined only in No. 2, before R 44656 21.1 ng/mL; R 6216 5.6 ng/mL; during R 44656 15.0 ng/mL; R 6216 4.4 ng/mL).

DISCUSSION

Nossel et al[2] reported that epitopic differences in anti-FPA antibody specificities may account for differences in measurable FPA levels in plasma samples due to fibrinogen breakdown in vitro, eg, during dialysis of plasma samples. Generation, in vitro, of a fragment of the α-chain of fibrinogen (Aα1-23) containing the FPA moiety was thought to explain the discrepancies seen in values measured with two anti-FPA sera because similar discrepancies were observed in dialysates of streptokinase-treated plasma samples. Rapid removal of fibrinogen from samples of plasma, by precipitation with ethanol and centrifugation, effectively inhibited this proteolysis of fibrinogen in vitro and resulted in the observation of similar FPA levels in patients with two anti-FPA sera.[2]

In the present study, fibrinogenolysis in vitro was inhibited by precipitating plasma samples with polyethylene glycol within 30 minutes of venipuncture, followed by heating of the supernatant in a water bath at 100 °C. Despite these precautions, discrepancies between values measured with two anti-FPA sera (R 44656 and R 6216, developed in our laboratory) were observed that could not be explained by assuming the presence of (plasmin-induced?) fibrinogen fragments. The

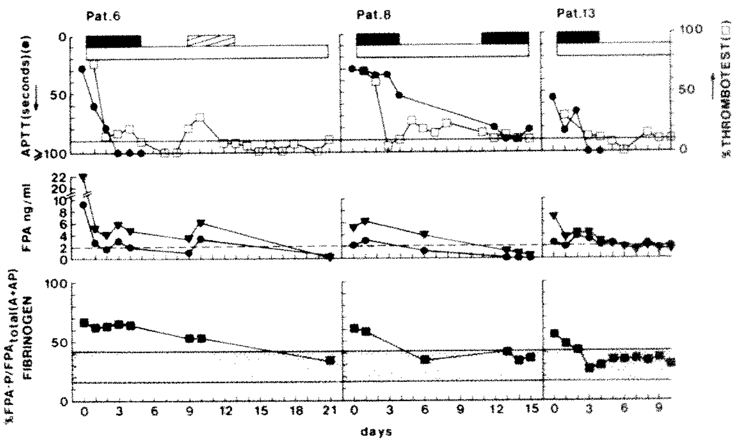

Fig 7. Effect of anticoagulant treatment on FPA levels and the FPA-P content of fibrinogen in three patients with venous thromboembolism (No. 6, 8, 13, Table 1). ■, intravenous heparin; □, acenocoumarol; ▨, chemotherapy (Adriamycin, cisplatinum). The upper limit of the therapeutic range with oral anticoagulants is indicated by a fully drawn line. A broken line denotes the upper limit of the normal range for FPA. Triangles represent FPA levels measured with R 44656, circles represent FPA levels with R 6216. The cross-hatched area represents the normal range for the FPA-P content in fibrinogen (mean ± 2 SD). In patient No. 8, heparin therapy was reinstituted because of symptoms of deep venous thrombosis.

Table 2. Effect of Alkaline Phosphatase on FPA Immunoreactivity

Patient	R 6216		R 44656	
	−	+	−	+
C.B.	1.1	3.3	3.4	3.4
C.N.	9.0	16.2	19.6	17.4
F.L.	2.0	4.2	5.0	4.5
P.M.	7.7	9.6	10.7	10.3
G.N.	2.5	5.6	5.0	4.8

Aliquots (0.05-mL) of PEG supernatant from five patients with venous thromboembolism were incubated in 0.450 mL assay buffer with (+) or without (−) 5 units alkaline phosphatase per milliliter for two hours at 37 °C. FPA concentrations were determined by radioimmunoassay with antisera R 6216 and R 44656. The enzyme alone had no effect on the assay. Purified FPA-P added to these PEG supernatants reacted equally with both sera after treatment.

following arguments substantiate this view. (1) The discrepancies remained on treatment with thrombin (not shown). (2) Such fibrinogen fragments are anticipated to react better with R 6216 than with R 44656. (3) The dissimilar reactivity of these antisera with patient's material was abolished by treatment with alkaline phosphatase (Table 2). (4) HPLC analysis, combined with treatment with alkaline phosphatase, of purified peptides and patients' samples indicated that the phosphorylated form of FPA accounted for the observed dissimilar reactivity of the two sera.

Our results obtained with these two anti-FPA sera show for the first time that, in patients with venous thromboembolism, FPA circulates in both its unphosphorylated and its phosphorylated forms. This observation may be relevant for the sensitivity of FPA immunoassays because, as reported in this article, polyclonal rabbit-anti-FPA sera, prepared according to routine protocols, may react differently with these two forms of the peptide. A difference in t½ of these two forms of the peptide seems unlikely in view of the prompt decline of FPA values with both antisera on intravenous heparin infusion (Fig 2).

As to the high FPA-P content in fibrinogen in patients with venous thromboembolism and elevated FPA levels (Table 1), the present study provides no evidence that these results are representative for the larger group of patients depicted in Fig 1. There can be no doubt, however, that highly phosphorylated fibrinogen can appear in the circulation in patients with venous thromboembolism. The appearance of this fibrinogen correlated with the presence of increased concentrations of FPA (Table 1 and Fig 7). That this correlation was not perfect can possibly be accounted for, at least partially, by the different t½s of fibrinogen (in the order of days)[17] and fibrinopeptide A (in the order of minutes).[2] This difference can manifest itself during anticoagulant therapy (Fig 7). Although adequate anticoagulation (as judged by APTT or thrombotest values) eventually led to a normalization of both FPA levels as well as the extent of phosphorylation of fibrinogen, this effect occurred at a slower pace than anticipated in one of the patients with a coexistent malignancy (patient 6, Fig 7). This observation appears to be in agreement with what has been observed by others for FPA levels in such patients.[18]

An interesting question is why the percentage of phosphorylated FPA in fibrinogen as well as free A-peptide material in samples of clinical blood may be as high as 80% of the total A-peptide material. Shainoff et al[19] hypothesized that, in newly synthesized fibrinogen, the serine in FPA was phosphorylated and an increased fibrinogen turnover would lead to the appearance in plasma of fibrinogen with a high phosphate content. Our results fit within this hypothesis because coagulation activation may lead to an increase in the fibrinogen turnover rate.[17] Regardless of which factor(s) will govern the extent of phosphorylation of plasma fibrinogen, we can now identify two situations in humans in which an increased FPA-P content, and therefore increased phosphate content, of plasma fibrinogen[20] is found: in infants at birth[21] (especially preterm[22]) and in some patients with signs of thrombin activity in vivo.

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REFERENCES

1. Nossel HL, Younger LR, Wilner GD, Procupez T, Canfield RE, Butler VP Jr: Radioimmunoassay of human fibrinopeptide A. Proc Natl Acad Sci USA 68:2350, 1971

2. Nossel HL, Yudelman I, Canfield RE, Butler Jr VP, Spanondis K, Wilner GD, Qureshi GD: Measurement of fibrinopeptide A in human blood. J Clin Invest 54:43, 1974

3. Gerrits WBJ, Flier OThN, van der Meer J: Fibrinopeptide A immunoreactivity in human plasma. Thromb Res 5:197, 1974

4. Nossel HL, Ti M, Kaplan KL, Spanondis K, Soland T, Butler VP: The generation of fibrinopeptide A in clinical blood samples. Evidence for thrombin activity. J Clin Invest 58:1136, 1976

5. Cronlund M, Hardin J, Burton J, Lee L, Haber E, Block KJ: Fibrinopeptide A in plasma of normal subjects and patients with disseminated intravascular coagulation and systemic lupus erythematosus. J Clin Invest 58:142, 1976

6. Yudelman IN, Nossel HL, Kaplan KL, Hirsh J: Plasma fibrinopeptide A levels in symptomatic venous thromboembolism. Blood 51:1189, 1978

7. Peuscher FW, van Aken WG, Flier OThN, Stoepman-van Dalen EA, Cremer-Goote THM, van Mourik JA: The effect of anticoagulant treatment measured by fibrinopeptide A (fpA) in patients with venous thromboembolism. Thromb Res 18:33, 1980

8. Blombäck B, Blombäck M, Edman P, Hessel B: Human fibrinopeptides. Isolation, characterization and structure. Biochim Biophys Acta 115:371, 1966

9. Goodfriend TL, Levine L, Fasman GD: Antibodies to bradykinin and angiotensin: A use of carbodiimides in immunology. Science 144:1344, 1964

10. Ruyven-Vermeer IAM, van Nieuwenhuizen W: Purification of rat fibrinogen and its constituent chains. Biochem J 169:653, 1978

11. Canfield RE, Dean J, Nossel HL, Butler Jr VP, Wilner GD: Reactivity of fibrinogen and fibrinopeptide A containing fibrinogen fragments with antisera to fibrinopeptide A. Biochemistry 15:1203, 1976

12. Merskey C, Kleiner GJ, Johnson AJ: Quantitative estimation

of split products of fibrinogen in human sera, relation to diagnosis and treatment. Blood 28:1, 1966

13. Godal MC, Abildgaard U: Gelation of soluble fibrin in plasma by ethanol. Scand J Haematol 3:342, 1966

14. Spackman DH, Stein WH, Moore S: Automatic recording apparatus for use in the chromatography of amino acids. Anal Chem 30:1190, 1958

15. Kehl M, Lottspeich F, Henschen A: Analysis of human fibrinopeptides by high-performance liquid chromatography. Hoppe-Seyler's Z Physiol Chem 362:1161, 1981

16. Koehn JA, Canfield RE: Purification of human fibrinopeptides by high-performance liquid chromatography. Anal Biochem 116:349, 1981

17. Harker LA, Slichter SJ: Platelet and fibrinogen consumption in man. N Engl J Med 287:999, 1972

18. Yudelman I, Greenberg J: Factors affecting fibrinopeptide A levels in patients with venous thromboembolism during anticoagulant therapy. Blood 59:787, 1982

19. Shainoff JR, Dyment PG, Hoffman GC, Bumpus FM: Fibrinopeptide composition in relationship to age of fibrinogen. Circulation 46:52, 1972 (suppl 2) (abstr)

20. Seydewitz HH, Kaiser C, Rothweiler H, Witt I: The location of a second in vivo phosphorylation site in the Aα chain of human fibrinogen. Thromb Res 33:487, 1984

21. Witt I, Müller H: Phosphorus and hexose content of human foetal fibrinogen. Biochim Biophys Acta 22:402, 1970

22. Galanakis DK, Martinez J, McDevitt C, Miller F: Human fetal fibrinogen: Its characteristics of delayed fibrin formation, high sialic acid and AP peptide content are more marked in preterm than in term samples, in Mosesson MW, Doolittle RF (eds): Molecular Biology of Fibrinogen and Fibrin. Ann NY Acad Sci 408:640, 1983

Analysis of Elevated Fibrin(ogen) Degradation Product Levels in Patients With Liver Disease

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Plasma and serum from patients with liver disease and elevated fibrin(ogen) degradation product (FDP) levels as measured by latex agglutination were analyzed by immunoblotting to characterize the FDP in these patients. An antihuman fibrinogen antibody was used that recognizes fibrinogen, fibrin monomer, soluble high molecular weight fibrinogen and fibrin polymers, as well as high molecular weight cross-linked degradation fragments, and the smaller fragments X, Y, D-dimer, D, and E. The analytic procedures were validated with plasma and serum from patients known to have intravascular fibrinolysis associated either with disseminated intravascular coagulation (DIC) or with thrombolytic therapy. The samples demonstrated a spectrum of plasmin degradation fragments on the immunoblots. Twenty-eight of 35 patients with liver disease (80%) had no evidence of plasmin degradation fragments in their plasma or serum. The cause of the elevated FDP levels as measured by latex agglutination was thought to be fibrin monomer or unclottable fibrinogen that was retained in the sera of some of these patients. Seven patients (20%) were found to have circulating plasmin degradation fragments. In addition to liver disease, however, these patients all had an illness (sepsis, shock, and pancreatic carcinoma) independently associated with intravascular coagulation and fibrinolysis. Three patients who lacked plasmin fragments also had pancreatic carcinoma or sepsis. The two groups of liver disease patients could not be clearly differentiated on the basis of clinical or laboratory evidence, but the blotting procedure proved to be a useful discriminator.

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ACQUIRED ABNORMALITIES of fibrinogen function are common in association with liver disease and have been reported in as many as 80% to 90% of patients with cirrhosis, chronic active liver disease, and hepatic failure.[1] A high incidence has also been observed in patients with hepatoma[2] and metastases to the liver.[1] The fibrinogen dysfunctions seen in these patients probably represent a heterogeneous group of disorders with multiple pathogenetic mechanisms that have not yet been fully elucidated. Fibrinogen dysfunction is usually manifested by prolonged thrombin and reptilase times and by abnormal polymerization of fibrin monomers.[3] The fibrinogen concentration is variable and may be low, normal, or high, and increased fibrinogen-fibrin degradation product (FDP) levels are frequently reported.[4-6] "Intravascular coagulation of liver disease" has become a common diagnosis in patients with liver disease and elevated FDP levels.[7]

Elevated FDP levels in liver disease patients with fibrinogen abnormalities raise questions concerning the biochemical characteristics of the FDP and the role of fibrinogenolysis and fibrinolysis in these patients. This is particularly important because an increased FDP level is often used as a primary criterion for a diagnosis of intravascular coagulation in liver disease. However, the poorly clottable character of fibrinogen in these patients may result in fibrin monomer or unclotted fibrinogen remaining in the serum and thus generating false-positive levels of FDP, as measured by the usual latex agglutination or staphylococcal clumping procedures.

The study described in this report focuses on characterizing FDP in liver disease patients. Plasma and serum samples from patients were analyzed for the presence of specific fibrinogen- or fibrin-derived degradation products by immunoblotting with antifibrinogen antibody. Application of this method to plasma and serum is a relatively new procedure that was validated by analysis of samples from patients with clear evidence of intravascular coagulation. With these procedures it was demonstrated that a high percentage of patients with liver disease do not have circulating fibrinogen or fibrin degradation fragments. The smaller number of patients with demonstrable circulating fragments also had a second condition clearly and independently associated with fibrin(ogen)olysis. These observations are clinically relevant for the treatment of these patients and also contribute to a definition of the pathogenesis of fibrinogen dysfunction in liver disease.

MATERIALS AND METHODS

Patient population. During an initial study to test the immunoblotting procedure, frozen serum and/or plasma samples were retrieved from the clinical hematology laboratory from Beth Israel Hospital (adult) patients with elevated FDP levels as measured by latex agglutination who also had a clinical picture suggestive of disseminated intravascular coagulation (DIC), including elevations of prothrombin time, partial thromboplastin time and/or thrombin time, and depression of platelets and fibrinogen, in the setting of an underlying disease frequently associated with DIC (Table 1).

After establishment of the sensitivity and specificity of the technique when applied to human serum and plasma, a prospective study of hospitalized patients with liver disease and elevated FDP levels as measured by latex agglutination was initiated. Throughout a 2-year period, consecutive serum samples showing elevated FDP levels and/or simultaneously drawn citrated plasma were retrieved from the clinical hematology laboratory. Each patient's record was reviewed in detail for historical, clinical, and laboratory evidence for the diagnosis of liver disease as well as for other relevant clinical data, including the presence of bleeding, thrombosis, or abnormal coagulation test results, and finally for coincident clinical conditions predisposing to DIC, including malignancy, shock, aneurysm, or

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Table 1. Patients With DIC and With Fragments Observed by Blotting

Patient No.	Diagnosis	FDP (μg/mL)	Fibrinogen* (mg/dL)	Platelet Count (× 10³ μL)
1	Pancreatic carcinoma	>320	120	94
2	Adenocarcinoma of lung	80–160	110	154
3	Acute promyelocytic leukemia	ND	90	28
4	Sepsis	>320	180	60
5	Cardiogenic shock	>320	63	272
6	Placental abruption	160–320	51	150
7	Streptokinase infusion	>320	<15	405

Abbreviation ND, not determined.

*Fibrinogen refers to clottable fibrinogen measured chronometrically.

sepsis. Therapeutic regimens were also recorded, including administration of blood products and/or medications.

Thirty-five patients with liver disease were studied (23 female, 12 male, aged 24 to 90 years). Criteria for liver disease included biopsy/autopsy diagnosis or abnormal liver function tests with a well-documented history of toxic exposure (ethanol/drug) or metastatic carcinoma. A clinical diagnosis of cirrhosis was accepted on the basis of long-standing jaundice, unequivocal signs of ascites or portal hypertension, and biochemical evidence of liver failure. Patients with abnormal liver function tests of brief duration (<1 week) and of unclear cause were excluded (although they would have strengthened the reported findings). Four patients with acute liver disease of >1 week's duration were included (nos. 26 to 28, and 32). Pregnant patients were also excluded. The decision to measure the FDP level in a patient was exclusively that of the patient's physician and generally represented the physician's suspicion that the patient had DIC.

Processing of blood samples. Plasma samples were prepared from normal or patient blood collected in 1/10 volume 3.8% trisodium citrate, centrifuged at 1000 g at 0 to 4 °C for 15 minutes, and stored at −20 °C for one to seven days and at −70 °C thereafter. Serum samples were prepared from normal or patient blood collected into 1,800 U/mL soy bean trypsin inhibitor and 10 U/mL bovine thrombin and clotted for 60 minutes at 37 °C. Samples were then centrifuged for five minutes at 1000 g and the serum removed and frozen in the same manner as the plasma samples.

Coagulation testing. Thrombin times were performed by adding 0.2 mL citrated plasma to 10 units of thrombin and recording the clotting time in a fibrometer.[8] Clottable fibrinogen was measured chronometrically by addition of dilutions of plasma to excess thrombin; the fibrinogen level was determined from a standard curve of known fibrinogen concentrations.[9] Fibrinogen levels were also measured immunochemically by radial immunodiffusion (M-Partigen plates, Calbiochem-Behring Corp, La Jolla, Calif). Latex agglutination of fibrinogen-related material was measured by the Thrombo-Wellcotest (Wellcome Reagents, Research Triangle Park, NC).[10] Platelet counts were determined with a Coulter Model S-Plus (Coulter Electronics, Hialeah, Fla) for counts greater than 50,000/μL or with phase microscopy for lower counts.

Immunoelectroblotting. Plasma or serum samples (1 μL) were prepared for electrophoresis in buffer containing 2% (wt/vol) sodium dodecyl sulphate (SDS), 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 0.001% bromophenol blue and subjected to electrophoresis on either 7.5% or 5% SDS polyacrylamide slab gels with a 3% stacking gel[11] for two hours at 45 mA. The separated proteins were transferred to nitrocellulose paper by a modification of the method of Towbin et al.[12] A constant voltage of 20 v was applied for 18 hours. The nitrocellulose paper was removed from the apparatus, preincubated with 3% hemoglobin in Tris-saline buffer (20 mmol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl) for one hour, incubated with ^{125}I-rabbit antihuman fibrinogen IgG (0.5×10^6 cpm/mL) in 3% hemoglobin-Tris-saline for one hour at room temperature, and finally washed in Tris-saline buffer containing 0.05% Tween 20 (Sigma, St Louis). The nitrocellulose was air dried and autoradiographs prepared using Kodak X-Omat AR-2 and a DuPont Cronex Lightning Plus intensifying screen at −70 °C.

Rabbit-antihuman fibrinogen IgG (100 μg) (Accurate Chemical, Westbury, NY) was iodinated with 2 mCi Na ^{125}I (New England Nuclear, Boston) using IODO-BEADS (Pierce Chemical CO, Rockford, Ill).[13] Iodinated IgG (specific activity approximately 5.5×10^7 cpm/mg) was stored at 4 °C in Tris-saline with 0.02% azide.

Purified human fibrinogen was iodinated by published methods[14] and had a specific activity of 3.0×10^8 cpm/mg.

RESULTS

Initial experiments were performed to examine the specificity of the antibody. The iodinated rabbit-antihuman fibrinogen IgG, when tested by immunoblotting, reacted with a protein in plasma that comigrated with fibrinogen and was completely removed by clotting. Furthermore, this antibody did not react with fibronectin or von Willebrand protein. Immunoblotting of plasma and serum samples from patients with clinical DIC (Table 1) as well as normal adults was performed using the iodinated antihuman fibrinogen. Figure 1 shows the spectrum of results obtained. Lanes 1 to 3 and 5 to 8 are plasma samples, lane 4 is serum, and lanes 9 and 10 are controlled plasmin digests of purified fibrinogen and cross-linked fibrin that were used as standards. Lane 9 (fibrinogen digest) contains fragments X, Y, D, and E as expected. Lane 10 (cross-linked fibrin digest) contains early high molecular weight fragments of cross-linked fibrin (mo-

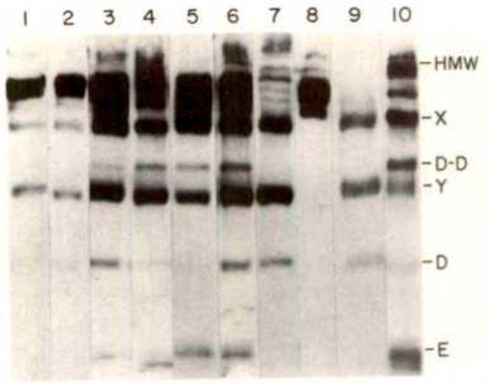

Fig 1. Detection of fibrinogen fragments in patient samples. Samples (1 μL) of human plasma (lanes 1 to 3 and 5 to 8) or serum (lane 4) were analyzed by SDS polyacrylamide (5%) gel electrophoresis, transferred to nitrocellulose, and reacted with ^{125}I-antihuman fibrinogen IgG. Immunoreactive proteins were detected by autoradiography. Patient diagnoses included pancreatic cancer (lane 1), adenocarcinoma of the lung (lane 2), acute promyelocytic leukemia (lane 3), sepsis (lane 4), cardiogenic shock (lane 5), and placental abruption (lane 6). The patient in lane 7 had received streptokinase therapy. Lane 8 is a pool of normal plasmas. Markers are degradation products prepared by treatment of purified fibrinogen (lane 9) or fibrin (lane 10) with plasmin (0.5 U/mL; 30 minutes, 37 °C).

lecular weight greater than that of fibrinogen), smaller cross-linked fragments (DY) that migrate in the fragment X region, and fragment D-dimer clearly showing that the fibrin had been covalently cross-linked by factor XIIIa. Smaller fragments in which the cross-links had been removed (Y and trace D) and fragment E were also observed, as expected. Plasma samples from patients with pancreatic carcinoma (lane 1) and adenocarcinoma of the lung (lane 2) clearly showed fibrinogen and fragments X and Y only, indicating that these were degradation products of fibrinogen or non–cross-linked fibrin. Plasma samples from patients with acute promyelocytic leukemia (lane 3) and placental abruption (lane 6) contained large amounts of high molecular weight material and D-dimer as well as the smaller fragments. A serum sample from a patient with sepsis (lane 4) had the same fragment pattern. This pattern is clearly indicative of plasmin degradation of cross-linked fibrin in these patients. The pattern in a plasma sample from a patient in cardiogenic shock (lane 5) was similar, with a clear D-dimer band indicating degradation of cross-linked fibrin. Very high molecular weight material was not seen in this patient, possibly indicative of a later, more advanced stage of fibrinolysis in which these fragments had already been cleaved. Lane 7 contains plasma from a patient treated with streptokinase for pulmonary embolism. This sample consists primarily of the degradation products of fibrinogen, as would be expected in a systemic lytic state. There is also some high molecular weight material, possibly indicating early lysis of the thrombus. Normal pooled plasma (lane 8) contains mainly monomeric fibrinogen plus a small amount of higher molecular weight material, probably representing the polymer in normal plasma described by Connaghan et al.[15]

The results in Fig 1 show that a variety of fragment patterns can be observed in patients with ongoing plasmin degradation, reflecting the principal substrate(s)—fibrinogen, non–cross-linked fibrin, cross-linked fibrin, or some combination of these. It should also be noted that D-dimer could be clearly resolved in this gel electrophoretic system and used as an index of cross-linked fibrin degradation. These results agree with those recently described by Connaghan et al[15] who also used immunoblotting of patient plasma and serum but a different electrophoretic gel system to separate fragments.

Experiments were carried out that demonstrated that fragments found in patient sera/plasmas were not generated in or removed from samples by prolonged storage (at least 24 months) at −70 °C or repeated freeze-thawing (four times). Additional studies (Fig 2) were carried out to estimate the efficiency with which ^{125}I-fibrinogen and its plasmin-generated fragments transfer from polyacrylamide gels (lanes 1 to 3) to nitrocellulose paper (lanes 4 to 6). Untransferred proteins are shown in lanes 7–9. Intact fibrinogen and fragment X did not transfer completely, but fibrinogen fragments (Y, D, E) did. In other experiments it was determined that when the plasma fibrinogen concentration (measured functionally and immunochemically) was 50 μg/mL, the fibrinogen band was readily apparent after transfer and blotting, FDP levels in the range of 10 to 40 μg/mL were also readily visualized.

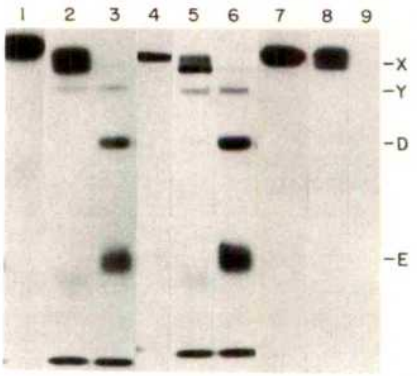

Fig 2. Transfer efficiency of intact fibrinogen and fibrinogen fragments to nitrocellulose. ^{125}I-fibrinogen was incubated without plasmin (lanes 1, 4, 7) or with plasmin, either 0.1 U/mL (lanes 2, 5, 8) or 0.4 U/mL (lanes 3, 6, 9) for one minute at 37 °C. Samples containing equivalent amounts of radioactivity were analyzed on an SDS polyacrylamide gel (7.5%) (lanes 1 to 3). Identical samples were applied to an equivalent gel and proteins subsequently transferred to nitrocellulose paper (lanes 4 to 6). The proteins that did not transfer from the SDS polyacrylamide gel are also shown (lanes 7 to 9). Radioactive proteins were detected by autoradiography.

Plasma and serum samples from 35 patients with documented disease were evaluated by immunoblotting. As shown in Table 2, 28 patients showed no evidence of degradation fragments despite the fact that they had apparently elevated FDP levels as measured by latex agglutination (range, 10 to 640 μg/mL). The platelet count and fibrinogen concentration were also very variable, with both ranging from very low to above normal. Thrombin times were prolonged in most patients tested. There was no correlation between platelet count, fibrinogen concentration, thrombin time, or apparent FDP level. Trace amounts of high molecular weight fibrinogen-related material, probably containing soluble fibrinogen or fibrin polymers, were observed in the plasmas of patients 2 and 3 but not in the remaining patients.

The source of elevated FDP levels as measured by latex agglutination for the patients in Table 2 was thought to be primarily fibrin monomer or unclottable fibrinogen, which was retained in several of the samples. This is illustrated for patient 20 who had an elevated fibrinogen level (500 mg/dL functional and 654 mg/dL immunochemical) and also had an FDP level as measured by latex agglutination of 160 to 320 μg/mL. On consecutive days repeated plasma and serum samples from the patient gave similar results. When analyzed by immunoblotting, his plasma showed a fibrinogen band only, with no evidence of degradation fragments or high molecular weight material (Fig 3, lane 1). However, a persistent band with the molecular weight of fibrin monomer or fibrinogen (lane 2) was repeatedly observed in his serum, despite appropriate collection procedures. The control serum (lane 3) contained no fibrinogen-related material. The material in the patient's serum was probably fibrin monomer that was retained because of the abnormal polymerization properties of the patient's fibrinogen. After storage at −70 °C for several months, the level of the serum material decreased. At

Table 2. Patients With Liver Disease but No Fragments Observed by Blotting

Patient No.	Liver Diagnosis	Secondary Diagnosis	FDP‖ (μg/mL)	Fibrinogen* (mg/dL)	Thrombin Time (s)	Platelet Count (K/μL)
1	Cirrhosis†	chr act hepatitis	40–80	212	ND	167
2	Cirrhosis†	chr act hepatitis	10–40	200	16.1/12.0	42
3	Cirrhosis	chr act hepatitis	10–40	260	ND	192
4	Cirrhosis†	chr act hepatitis	40–80	140	25.3/18.3	ND
5	Cirrhosis†	chr act hepatitis	10–40	159	150/19.7	53
6	Cirrhosis†	pneumonia	40–80	335	35.5/18.3	83
7	Cirrhosis	hepatoma‡	40–80	320	18.3/16.8	5
8	Cirrhosis†	pancreatic mets	40–80	540	18.9/16.9	264
9	Cirrhosis†	α-1-antitrypsin deficiency	40–80	130	ND	217
10	Cirrhosis†	urine infection	10–40	160	23.9/16.4	91
11	ETOH§	respiratory arrest	10–40	160	ND	70
12	ETOH	peritonitis	40–80	240	16.8/11.6	215
13	ETOH	pneumonia	40–80	1,000	25.4/18.2	628
14	ETOH	epistaxis	40–80	69	ND	34
15	ETOH	myelofibrosis/leukemia	10–40	500	13.4/11.8	32
16	Myelofibrosis†	pulmonary hemorrhage	10–40	78	20.7/19.4	12
17	Hemachromatosis†	congestive failure	40–80	140	100/17.7	31
18	Hemosiderosis†	empyema	320–640	380	19.3/17.3	NL
19	Hepatoma‡	pleural effusion	10–40	320	41.9/18.6	123
20	Melanoma mets	venoocclusive disease	160–320	500	36.4/11.4	51
21	Breast ca mets	venoocclusive disease	10–40	320	27.5/17.4	10
22	Colon ca mets	—	10–40	700	ND	ND
23	Breast ca mets†	sepsis	40–80	600	24.8/17.2	28
24	Breast ca mets	sepsis	80–160	320	21.7/19.6	22
25	Pancreas ca mets†	biliary obstruction	10–40	360	27.1/19.9	254
26	Hepatic infarct†	bowel infarct	40–80	410	ND	ND
27	Toxic/metabolic	hemolytic/uremic syndrome	10–40	270	17.6/13.3	105
28	Toxic/metabolic†	gastrointestinal bleed	40–80	153	33.4/19.1	54

Abbreviations: chr, chronic; act, active; ca, cancer; mets, metastases, ND, not determined; NL, normal on platelet smear.

*Fibrinogen refers to clottable fibrinogen measured chronometrically.

†Confirmed by biopsy/autopsy.

§ETOH refers to patients with a history of chronic alcohol abuse who had abnormal livers as shown by various noninvasive studies but on whom no liver biopsies had been performed.

‡Hepatoma was diagnosed on the basis of a characteristic radiographic picture with a markedly elevated α-fetoprotein in the setting of newly acquired ascites.

‖FDP was measured by latex agglutination.

Fig 3. Immunoreactive material present in the serum of patient 20 with positive latex agglutination for fibrinogen-related material. Samples (1 μL) of patient plasma (lane 1), patient serum (lane 2), and control serum (lane 3) were analyzed as described in Fig 1. Patient serum showed a persistent band of fibrinogen-related materials not seen in control serum.

that time latex agglutination demonstrated a decrease to 40 to 80 μg/mL, and repeat immunoblotting showed the serum fibrinogen–related band to be less prominent. The instability of this material to storage at −70 °C, as compared with the marked stability of fibrinogen and degradation fragments, also is in agreement with this conclusion that this material was fibrin monomer that did not polymerize normally.

In contrast to the 28 liver disease patients who only had evidence of fibrin monomer or unclotted fibrinogen in their sera, seven patients were found to have degradation fragments as shown by immunoblotting (Table 3). Each of these patients also had a second condition independently associated with DIC.

DISCUSSION

Since the time of Goodpasture's early observation that some patients with chronic liver disease had shortened euglobulin lysis times,[16] there has been considerable interest and debate concerning the contribution of fibrinolysis to the coagulopathy of liver disease. A number of important observations have been made, but many of the earlier studies were

Table 3. Patients With Liver Disease and Degradation Fragments Observed by Blotting

Patient No.	Liver Diagnosis	Secondary Diagnosis	FDP (μg/mL)	Fibrinogen* (mg/dL)	Thrombin Time (s)	Platelet Count ($\times$ 10^3 μL)
29	ETOH†	sepsis	10–40	400	72.3/18.5	18
30	ETOH	shock	40–80	285	26.8/16.8	183
31	Cirrhosis	sepsis	160–320	335	29.6/18.6	79
32	Hepatitis‡	sepsis	40–80	198	22.4/18.9	12
33	Hemosiderosis‡	sepsis	80–160	235	23.6/18.1	18
34	Gastric ca mets‡	sepsis	40–80	490	ND	98
35	Pancreas ca mets‡	pancreatic ca	10–40	70	ND	21

Abbreviations: mets, metastases; ca, cancer; ND, not determined.

*Fibrinogen refers to clottable fibrinogen measured chronometrically.

†ETOH refers to patients with a history of chronic alcohol abuse who had abnormal livers as shown by various noninvasive studies but on whom no liver biopsies had been performed.

‡Confirmed by biopsy/autopsy.

complicated by technical problems and insensitive assays. Earlier studies with labeled fibrinogen suggested increased fibrinogen turnover.[17-19] In one study it was also found that heparin infusion prolonged the half-life of labeled fibrinogen in these patients.[17] However, this work has been criticized by other investigators[20] who suggested that extravasation of labeled fibrinogen into ascites could account for an apparent increase in fibrinogen catabolism and that the heparin effect was uninterpretable because the labeled fibrinogen was measured at a time when the amount remaining was too close to background.

Other relevant observations concern measurements of fibrinolytic system components as an index of activation of the system and measurements of FDP as an index of plasmin activity. Reduction in antiplasmin levels and elevation of plasminogen activator levels have been observed.[21-23] Marongiu et al reported reduced antiplasmin and increased fibrinopeptide A levels in 26 cirrhotic patients, but found no corresponding alteration in FDP and fibrinogen levels.[24] Das and Cash demonstrated an exaggerated fibrinolytic response to exercise in patients with cirrhosis, but also noted no correlation between increases in plasminogen activator and serum FDP levels following exercise in these patients.[25] Merskey et al found that nine of 21 patients (43%) with hepatic necrosis or cirrhosis had elevated FDP levels as shown by the tanned red cell hemagglutination inhibition immunoassay.[4] There are also additional reports of increased serum FDP levels in liver disease patients.[5,6,26] However, other studies have found that FDP levels were not uniformly increased in these patients.[25,27] Studies in which the FDP in liver disease patients have been characterized have not been reported. Consequently, the role of plasmin degradation in the pathogenesis of fibrinogen dysfunction in liver disease is unclear.

In the study reported here, 35 patients with clearly documented liver disease and elevated FDP levels as shown by latex agglutination were investigated. Fibrinogen-related material in patient plasma or serum was identified by immunoblotting. The antibody to human fibrinogen that was used is specific for fibrinogen and fibrinogen-derived products, including fibrin monomer, soluble fibrinogen, and fibrin polymers, and for the plasmin degradation fragments derived from fibrinogen, non–cross-linked fibrin, and cross-linked fibrin. This procedure has been used to identify plasmin cleavage products of fibrinogen and fibrin in samples from more than 60 patients with ongoing fibrinolysis, seven representative cases of which are described in Fig 1 and Table 1. Degradation fragments could be clearly identified in appropriate patient plasma or serum containing as little as 10 to 40 μg/mL FDP as measured by latex agglutination.

Analysis of patients with liver disease is shown in Fig 3 and Tables 2 and 3. Of the 35 patients, 28 had no evidence for degradation fragments of fibrinogen or fibrin, including X, Y, D, E, or D-dimer (Table 2). Patients 2 and 3 with cirrhosis and chronic active hepatitis had trace amounts of high molecular weight fibrinogen-related material in their plasmas but not in their sera, which probably represented soluble polymers of fibrinogen or fibrin. Patient 3 also had hemophilia A. In some of these 28 patients, the reported elevated FDP level was found to be caused by fibrinogen-related antigen in their sera that had a molecular weight identical to fibrinogen and fibrin monomer. This material was clearly not a plasmin degradation fragment of fibrinogen or fibrin. It was most evident in fresh serum samples and was greatly diminished after the samples had been frozen and thawed. This behavior is more characteristic of fibrin monomer than fibrinogen. At very low concentrations fibrin monomer can be maintained in solution, but upon freezing and thawing precipitation occurs. Because of this observation and because fibrin monomer polymerization is often defective in liver disease patients, it is probable that the serum material from these patients was fibrin monomer. In addition, a second possibility of dysfunctional, unclottable fibrinogen cannot be ruled out. However, the significant point is not whether the patient sera contained fibrin monomer or fibrinogen, but rather that there were not plasmin degradation fragments in these sera. Hence, it can be concluded that fibrinolysis was not occurring in these liver disease patients.

Plasminolytic activity was documented in seven patients (20%) in this study (Table 3). These patients did not differ from the others in the extent of liver disease, nor were their coagulation parameters distinctive. However, these patients all had a second illness (sepsis, shock, or pancreatic carcinoma) frequently associated with intravascular coagulation and fibrinolysis, independent of liver disease. Three of the patients without evidence of fibrinolysis also had sepsis or pancreatic carcinoma.

The results of this study indicate that plasmin degradation of fibrinogen and fibrin is not a frequent complication in patients with liver disease who have elevated FDP levels detected by the usual laboratory tests. These patients may have dysfunctional fibrinogen, resulting in unclotted fibrinogen-related material or fibrin monomer remaining in their sera. A smaller number of patients with liver disease do have clear evidence for plasmin activity. These patients also appear to have a secondary illness often associated with intravascular coagulation. Differentiation between the two groups of patients may not be simple since the clinical and laboratory parameters may be the same. In such cases the immunoblotting procedure described here may be helpful. Similar techniques have demonstrated usefulness in detailed analysis of other coagulopathies such as fibrinogenolytic afibrinogenemia[28] and thrombus formation.[15]

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REFERENCES

1. Francis JL, Armstrong DJ: Acquired dysfibrinogenemia in liver disease. J Clin Pathol 35:667, 1982

2. Gralnick HR, Givelber H, Abrams E: Dysfibrinogenemia associated with hepatoma. Increased carbohydrate content of the fibrinogen molecule. N Engl J Med 299:221, 1978

3. Lane DA, Sculley MF, Thomas DP, Kakkar VV, Woolf IL, Williams R: Acquired dysfibrinogenemia in acute and chronic liver disease. Br J Haematol 35:301, 1977

4. Merskey C, Kleiner GS, Johnson AJ: Quantitative estimation of split products of fibrinogen in human serum, relation to diagnosis and treatment. Blood 28:1, 1966

5. Hillenbrand P, Parbhoo SP, Jedrychowski A, Sherlock S: Significance of intravascular coagulation and fibrinolysis in acute hepatic failure. Gut 15:83, 1974

6. Gallus AS, Lucas CR, Hirsh J: Coagulation studies in patients with acute infectious hepatitis. Br J Haematol 22:761, 1972

7. Verstraete M, Vermylen J, Collen D: Intravascular coagulation in liver disease. Ann Rev Med 25:447, 1974

8. Davey FR: Blood coagulation and its disorders, in Henry JB (ed): Clinical Diagnosis and Management by Laboratory Methods (ed 16). Philadelphia, Saunders, 1979, p 1131

9. Brown B: Hematology: Principles and Procedures (ed 3). Philadelphia, Lea and Febiger, 1980, p 135

10. Allington MJ: Detection of fibrin(ogen) degradation products by a latex clumping method. Scand J Haematol 13:115, 1971 (suppl)

11. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

12. Towbin H, Staehlin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Nat Acad Sci USA 76:4350, 1979

13. Markwell MA: A new solid state reagent to iodinate proteins. I. Conditions for the efficient labeling of antigen. Anal Biochem 125:427, 1982

14. Kaminski M, McDonagh J: Studies on the mechanism of thrombin: Interaction with fibrin. J Biol Chem 258:10530, 1983

15. Connaghan DG, Francis CW, Lane DA, Marder VJ: Specific identification of fibrin polymers, fibrinogen degradation products and crosslinked fibrin degradation products and crosslinked fibrin degration products in plasma and serum with a new sensitive technique. Blood 65:589, 1985

16. Goodpasture EW: Fibrinolysis in chronic hepatic insufficiency. Bull Johns Hopkins Hospital 25:330, 1914

17. Tytgat GN, Collen D, Verstraete M: Metabolism of fibrinogen in cirrhosis of the liver. J Clin Invest 50:1690, 1971

18. Brodsky I, Siegel NH, Kahn SB, Ross EM, Petkov G: Simultaneous fibrinogen and platelet survival with ^{75}Se selenomethionine in man. Studies in diseases with normal coagulation and in hepatocellular disease with abnormal coagulation. Br J Haematol 18:341, 1970

19. Rake MO, Flute PT, Pannell G, William R: Intravascular coagulation in hepatic necrosis. Lancet 1:533, 1970

20. Straub PW: Diffuse intravascular coagulation in liver disease? Semin Thromb Hemost 4:29, 1977

21. O'Connell RA, Grossi CE, Rousselot LM: Role of inhibitors of fibrinolysis in hepatic cirrhosis. Lancet 2:990, 1964

22. Tytgat G, Collen D, DeVreker RA, Verstraete M: Investigations on the fibrinolytic system in liver cirrhosis. Acta Haematol (Basel) 40:265, 1968

23. Fletcher AP, Biederman O, Moore D, Alkjaersig N, Sherry S: Abnormal plasminogen-plasmin system activity (fibrinolysis) in patients with hepatic cirrhosis. Its cause and consequences. J Clin Invest 43:681, 1964

24. Marongiu F, Mamusa AM, Mamali G, Mulas G, Solinas A, Demelia L, Contu L: α_2 antiplasmin and disseminated intravascular coagulation in liver cirrhosis. Thromb Res 37:287, 1985

25. Das PC, Cash JD: Fibrinolysis at rest and after exercise in hepatic cirrhosis. Br J Haematol 17:431, 1969

26. Bergstrom K, Blömback B, Kleen G: Studies on the plasma fibrinolytic activity in a case of liver cirrhosis. Acta Med Scand 168:291, 1960

27. Clark RD, Gazzard BG, Lewis ML, Flute PT, Williams R: Fibrinogen metabolism in acute hepatitis and active chronic hepatitis. Br J Haematol 30:95, 1975

28. Budzynski AZ, Pandya BV, Rubin RN, Brizuela BS, Soszla T, Stewart GJ: Fibrinogenolytic afibrinogenemia after evenomation by Western Diamondback Rattlesnake (*Croatalus atrox*). Blood 63:1, 1984

Increased Red Blood Cell Deformability Due to Isoxsuprine Administration Decreases Platelet Adherence in a Perfusion Chamber: A Double-Blind Cross-Over Study in Patients With Intermittent Claudication

By Piet A.M.M. Aarts, Jan Dirk Banga, Hans C. van Houwelingen, Robert M. Heethaar, and Jan J. Sixma

Platelet transport towards the vessel wall is influenced by the hematocrit, red blood cell (RBC) size, and shape. Recent in vitro studies have indicated that RBC deformability may also influence platelet transport. The observation that isoxsuprine, a known vasodilating drug, caused increased RBC deformability in vitro and decreased platelet transport in vitro prompted us to study the effects of this drug in vivo. The study was performed in a double-blind cross-over study of isoxsuprine *v* placebo in ten patients with peripheral arterial insufficiency. RBC deformability was estimated from viscosity measurements using the blood viscosity equation of Dintenfass and expressed as T value. Platelet transport was studied in an annular perfusion chamber according to Baumgartner. Human umbilical arteries were used as blood vessels. Perfusion studies were performed with whole blood or with RBCs of the patients mixed with normal platelets and plasma at a standardized hematocrit and platelet count. An increase in RBC deformability concomitant with a decrease in platelet adherence was observed in patients on isoxsuprine with a drop in T value of ~0.06 (from 0.91 toward 0.86), and a concomitant decrease in platelet adherence of 20% to 40%. These observations differed significantly from the results in the placebo group and showed a significant group-period interaction at the cross-over of medication (analysis of variance). The effects on platelet adherence were observed at high vessel wall shear rate (1,800 s^{-1}) with perfusates consisting of patients' RBCs and donor plasma and platelets at standardized hematocrit and platelet count. No differences were observed under these conditions at a shear rate of 300 s^{-1}. When whole blood of patients was used, nonsignificant effect was observed at shear rates of 300 s^{-1} and 1,800 s^{-1}. This was probably caused by the added noise due to variations in hematocrit and platelet number. These data demonstrate that isoxsuprine increases RBC deformability, and they suggest the possibility of decreasing platelet-vessel wall interaction in vivo by manipulation of RBC deformability.

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ADHERENCE OF BLOOD platelets and subsequent aggregate formation on the subendothelium of the damaged vessel wall are fundamental events in thrombosis and hemostasis.[1,2] An in vitro perfusion chamber to study these phenomena under conditions of flow has been developed by Baumgartner and Haudenschild.[3] Previous studies using this technique confirmed the essential role of the red blood cells (RBCs) in this process, and showed that the hematocrit is one of the main factors influencing platelet adherence.[4] Recently, we reported that RBC size[5] and RBC deformability are also important,[6] although the effect of RBC size was more pronounced than was the effect of RBC deformability. However, RBC deformability may have greater clinical importance because it may be influenced by treatment, and therapeutic regimens may be devised that have an antithrombotic effect because of a decreased platelet transport towards the vessel wall by RBCs. Recent in vitro perfusion studies clearly showed that decreased RBC deformability (hardening) caused enhanced platelet adherence, whereas increased deformability caused a decrease in platelet adherence. Manipulation of RBC deformability was achieved by treatment of the RBCs in vitro with various chemicals.[6] One of these substances, isoxsuprine (Duvadilan) has been applied in clinical practice as a "vasodilating drug." More recent studies have indicated that isoxsuprine may decrease blood viscosity. These effects were found in vitro[7-9] and in vivo.[8-10] Our objective was to extend our observations to the in vivo situation, and we achieved it by oral administration of isoxsuprine in a double-blind cross-over study of 12 patients suffering from intermittent claudication who were examined for five months. During the study, perfusion experiments were performed, and the patients were subjected to clinical examination and laboratory blood tests.

MATERIALS AND METHODS

Patients and clinical examinations. The study group consisted of 12 men, aged 50 to 72 years (mean ± SD 64 ± 6 years) with peripheral arterial insufficiency. Vascular surgery had been performed in two of them, vascular surgery combined with lumbar sympathectomy in one, and lumbar sympathectomy alone in two. All 12 men had stable intermittent claudication, and further vascular reconstrictive surgery was not considered. All patients consented to cooperate in the study after being given detailed information. One of them died suddenly of a ruptured aneurysm three weeks after the start of the trial; another patient refused further participation in the study after three months because of upper abdominal discomfort. All patients were on oral anticoagulation with coumarin derivatives and continued to use this medication throughout the trial period.

The study period lasted 20 weeks and consisted of five episodes of four weeks. Clinical examinations including the completion of a standardized questionnaire and blood analysis were performed at the beginning and end of each episode. The first four weeks were considered a washout period in which all patients received placebo. The study period thereafter was divided in two periods of eight weeks. Each patient received either isoxsuprine or placebo during eight weeks and then switched to either placebo or isoxsuprine for the following eight weeks. Whether a patient first started with placebo and then isoxsuprine or the converse was selected at random. The medication consisted of identical capsules containing either 40 mg of isoxsuprine or placebo (courtesy of Duphar Nederland B.V., Amsterdam), taken orally two times daily throughout the study period. The testing procedure throughout the study period was as

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follows: (a) measurement of brachial and ankle systolic blood pressure and determination of the ankle-arm pressure ratio (ankle-arm pressure index) at rest and after maximal walking distance; (b) the maximal walking distance was established on a treadmill using the modified Bruce protocol[11]; (c) brachial and ankle systolic pressures and the pressure index were measured at one, three, five, and ten minutes after excercise.

The time schedule and the points at which clinical and laboratory examinations were performed are depicted in Fig 1 and were as follows: (a) treadmill tests and pressure measurements were performed the first and last day of the washout period and at the end of each eight-week period, during which the patient received either isoxsuprine or placebo according to a double-blind randomized cross-over schedule (points 1, 2, 4, and 6); (b) laboratory tests were performed at the beginning and end of each four-week period (points 1 through 6).

Viscosity measurements and determinations of RBC deformability from internal viscosity. The viscosity of plasma, blood, and RBC suspensions was measured with a Couette type viscosimeter Contraves LS 30 (Contraves A.G., Zurich) at a shear rate of 130 s^{-1} as previously described.[6] RBC deformability was determined according to Dintenfass.[12,13] An empirical relation concerning the viscosity of blood and RBC suspension under Newtonian flow conditions was described by Dintenfass as:

$$\mu_s = \mu_0 (1 - TH)^{-2.5}, \qquad [1]$$

where μ_s = viscosity of the suspension (mPas); μ_0 = viscosity of the suspending medium; H = hematocrit (vol/vol) and T is the so-called "Taylor factor," a dimensionless parameter, related to RBC deformability. Increasing values of T indicate decreased RBC deformability (increased rigidity) and vice versa. The T value estimated by measurement of viscosity and H of a RBC suspension can thus be used as a parameter for RBC deformability. Prerequisites for the validity of this relation are normal human cells mean cell volume (mcv) between 85 and 100 fL) and Newtonian behavior, or absence of RBC aggregation, which was achieved by measurement of RBC suspensions in buffer (absence of fibrinogen) at a shear rate of 130 s^{-1}. Differences in suspension viscosity caused by differences in RBC deformability were most pronounced at higher H values ($H > 0.60$); however, at H values >0.80, the couette flow is unstable. For this reason, we used suspensions of RBCs in isotonic saline (1 mL) with $0.60 < H < 0.80$, at a uniform shear rate of 130 s^{-1} (37 °C).

In a control experiment, using a Wells-Brookfield viscosity meter, a shear rate of 600 s^{-1} was compared with the shear rate of 130 s^{-1}, used as standard for the clinical studies. The absolute value of T decreased from 0.91 ± 0.02 at 130 s^{-1} to 0.80 ± 0.02 at 600 s^{-1}. The decrease by in vitro treatment with isoxsuprine was similar at both shear rates. Control experiments performed at a H of 0.40 demonstrated that the determination of T was independent of the RBC concentation.

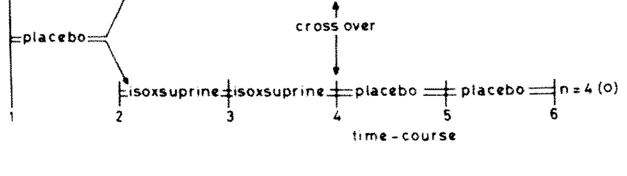

Fig 1. Medication schedule during the course of the study. The numbers 1 through 6 refer to test points. The length of the periods between two test points was four weeks. During the trial, two patients who belonged to the isoxsuprine–placebo group dropped out; therefore, this group consisted of four patients whereas the placebo–isoxsuprine group consisted of six patients.

Platelet adherence experiments. Platelet adherence studies were performed to find out whether changes in RBC deformability caused by drug treatment were large enough to affect platelet adherence and to have relevance for a thrombotic tendency.

Perfusion studies were carried out with the annular perfusion chamber of Baumgartner, using the steady flow system of Sakariassen.[14] Arteries were isolated from the human umbilical cord (obtained immediately after birth), stored in buffer (0.2 mol/L of Tris, pH 7.4), and used within 24 hours, after a one-hour treatment with 0.1 mmol/L of aspirin to inhibit prostacyclin production by the vessel wall. Perfusions were performed with whole blood of the patients and with recombined perfusates. These perfusates consisted of RBCs from the patient combined with plasma and platelets from a healthy donor with the same blood group. These two types of perfusion experiments were chosen so that we could discriminate between potential influences on platelet adherence: that of RBC deformability per se and that of action of isoxsuprine on platelets, plasma components, and subendothelium. Blood samples from patients and donors were drawn into one-ninth vol of 110 mmol/L of trisodium citrate. Platelet-rich plasma (PRP) was prepared from donor blood by centrifugation at 190 g for ten minutes (20 °C). The remaining blood was centrifuged at 3,000 g for 15 minutes (20 °C), to obtain platelet-poor plasma (PPP). The PRP and PPP were mixed to obtain standard PRP with 190,000 platelets per plasma. This standard PRP was mixed with washed, packed RBCs of the patients to obtain a standard H of 0.4. This standard H and platelet count was similar to those in previous studies.[5,6] Packed and washed RBCs of the patients were obtained by centrifugation of patients' whole blood for ten minutes at 3,000 g (20 °C). PPP was then removed, and two additional washes with isotonic saline containing 5 mmol/L of glucose were performed. The small buffy coat on top was removed carefully each time. Packing was done at 3,000 g for 15 minutes (20 °C).

Perfusions were performed at vessel wall shear rates of 300 s^{-1} and 1,800 s^{-1}. A vessel wall shear rate of 300 s^{-1} was obtained with a perfusion chamber with a diameter of 6.1 mm (effective annular width 1.05 mm) at a flow rate of 35 mL/min. To obtain a vessel wall shear rate of 1,800 s^{-1}, a perfusion chamber with a diameter of 5.0 mm (effective annular width 0.60 mm) and a flow rate of 100 mL/min was used. Wall shear rates were calculated from flow equations of a Newtonian fluid through an annular slit similarly to calculations in our previous experiments.[15] Recently, we used Laser-Doppler velocimetry to measure velocity profiles in the annular chamber from which vessel wall shear rates could be determined experimentally.[16] This study showed that measured velocity profiles and derived values for wall shear rate agreed fully with theory. Perfusions were performed at 37 °C for five minutes after prewarming at 37 °C for five minutes.

After perfusion, the vessel segments were fixed in 0.2 mol/L of phosphate buffer containing 2.5% glutaraldehyde and 2% osmium tetroxide and embedded in epon after dehydration as described by Baumgartner.[17] Thin cross-sections, 1 µm thick, were cut on an ultratome (type 8800, LKB, Bromma, Sweden) and stained with methylene blue and basic Fuchsin (Merck, Darmstad, F.R.G.).[18] Platelet adherence to the subendothelium was quantified by morphometry, based on counting 1,000 intersection points at a 10-µm distance, using a light microscope (Leitz-Wetzlar dialux 20 EB, E. Leitz GmbH, Wetzlar, F.R.G.) at 1,000× magnification according to Baumgartner.[13] Platelet interaction was subdivided into contact platelets, spread platelets, or aggregates (>10 µm high). Total platelet adherence was expressed as percentage of coverage.

Laboratory tests. On each test day, a set of routine laboratory tests were performed. Five milliliters of whole blood, anticoagulated with 2 mmol/L of potassium EDTA, was collected for determination of the hematocrit, mcv, hemoglobin concentration (Hb), leukocyte count (Coulter-counter, Model-S, Harpenden, England), and platelet count (Platelet Analyzer 810, Baker Diagnostics Ltd, Bethlehem).

Levels of β-thromboglobulin (β-TG) and platelet factor 4 in the plasma of the patients were determined with a β-TG radioimmunoassay (Amersham International plc, Amersham, England) and a PF-4 radioimmunoassay kit (Abbott Laboratories, North Chicago), respectively. Platelet aggregation by ADP was determined in a Payton Dual Channel Aggregation Module (Payton Associates, Inc, Scarborough, Canada) with 2.5 μmol/L and 5.0 μmol/L ADP (American Dade, Miami). Bleeding time was determined with Simplate-II device (General Diagnostics, Morris Plains, NJ).

Statistical analysis. All variables determined during the course of the study were tested. As a routine check, *t* tests between the groups (placebo–isoxsuprine and isoxsuprine–placebo) were performed at successive test points. However, the individual *t* tests at respective test points were not related to each other and were not adequate to detect significant effects of the medication during the time course of the study. A more suitable method to test for correlation of the variables with the medication schedule is analysis of variance in repeated measures, which was computed with a BMD 4V-program. Only the data of points 3, 4, 5, and 6 were used, because points 1 and 2 gave no information about medication effect. Differences between isoxsuprine and placebo should result in a significant group–period interaction. Moreover, the signs of the interaction effect should be in accordance with the cross-over after point 4 (it should be either $+ + - -$ or $- - + +$).

Correlation between variables was computed in two different ways. First, all 60 data points (ten patients at six points) were used to construct scatter diagrams and to compute and test the correlation coefficient. The disadvantage of this method is that correlation may be due to either patient or treatment effect. To eliminate the patient effect, we computed the measure of decrease in time of a variable X defind by $X_3 + X_4 - X_5 - X_6$ (X_i = measurement on point i), which yielded one figure per patient. A test for correlation between two parameters, X and Y, was performed by analysis of covariance, yielding information about whether correlation was due to the treatment or was also present within the two separate groups.

RESULTS

Viscosity measurements. Whole blood viscosity, plasma viscosity, and RBC deformability expressed as the mean value (±SD) for both groups during the course of the study are presented in Fig 2. RBC deformability showed significant changes congruent with the medication schedule. In addition, a significant group–period interaction was found by analysis of variance ($P = .006$). No significance was found for whole blood viscosity by BMD P4V; however, at test point 6, a significant difference (*t* test) occurred between isoxsuprine and placebo group ($P = .005$). However, when whole blood viscosity was normalized to a standard viscosity (a H of 0.4), a significant group–period interaction with a P value of .015 was found using equation 1. This is shown in Table 1. Plasma viscosity was constant and equal in both groups during the study.

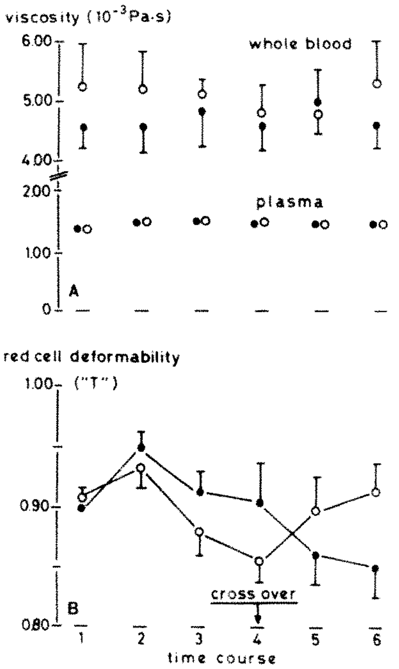

Fig 2. (A) Whole blood viscosity, plasma viscosity, and (B) RBC deformability during the course of the trial for both groups of patients [placebo–isoxsuprine group (●); isoxsuprine–placebo group (○)]. Only for RBC deformability was a significant group–period interaction congruent with medication found ($P = .006$); *t* tests at the separate test point showed a significant difference between the groups for whole blood viscosity at point 6 ($P = .05$) and for RBC deformability at point 4 ($P = .013$) and point 6 ($P = .003$).

Platelet adherence. The data on platelet adherence obtained in the various types of perfusion experiments during the course of treatment are listed in Table 2. A treatment effect with significant group–period interaction ($P = .017$) was seen in the perfusion experiments in which the RBCs of the patients were supplemented with plasma and platelets of normals at a wall shear rate of 1,800 s^{-1} (Fig 3). The platelet adherence in these experiments was significantly lower with the RBCs of patients who used the active drug. In the other types of perfusion experiments, no significant effect of medication was found.

In the perfusion experiments with whole blood from the patients at a wall shear rate of 1,800 s^{-1}, a large proportion of the vessel surface was covered with platelet aggregates (see

Table 1. Normalized Whole Blood Viscosity

Test Point	Time Course	Medication	Normalized Viscosity (± SD)
1	Start	Placebo	3.74 ± 0.28
		Placebo	3.75 ± 0.15
2	4 wk	Placebo	3.97 ± 0.40
		Placebo	4.14 ± 0.34
3	8 wk	Placebo	4.05 ± 0.28
		Isoxsuprine	4.08 ± 0.28
4	12 wk	Placebo	4.18 ± 0.90
		Isoxsuprine	3.76 ± 0.48
5	16 wk	Isoxsuprine	3.36 ± 0.20
		Placebo	3.75 ± 0.09
6	20 wk	Isoxsuprine	3.51 ± 0.24
		Placebo	4.05 ± 0.24

Whole blood viscosity was normalized from the actual hematocrit to a standard value of 0.4 using eq. 1: $\mu_s = \mu_0 (1\text{-}TH)^{-2.5}$. Significance for group–period interaction: $P = .016$.

Table 2. Platelet Adherence for All Types of Perfusion Experiments During the Study

			Whole Blood		Red Cells of Patients + Donor Plasma and Platelets	
Test Point	Time Course	Medication	1,800 s^{-1}	300 s^{-1}	1,800 s^{-1}	300 s^{-1}
1	Start	Placebo	73 ± 3 (37)	33 ± 3 (4)	68 ± 11 (6)	42 ± 7 (3)
		Placebo	69 ± 17 (33)	40 ± 9 (2)	51 ± 2 (2)	47 ± 3 (4)
2	4 wk	Placebo	70 ± 7 (22)	40 ± 8 (7)	76 ± 4 (4)	46 ± 11 (5)
		Placebo	78 ± 4 (20)	50 ± 6 (6)	74 ± 18 (9)	47 ± 13 (4)
3	8 wk	Placebo	63 ± 11 (16)	44 ± 7 (5)	65 ± 11 (7)	43 ± 6 (4)
		Isoxsuprine	63 ± 20 (4)	45 ± 18 (3)	58 ± 12 (4)	34 ± 7 (7)
4	12 wk	Placebo	60 ± 4 (7)	34 ± 4 (6)	54 ± 6 (5)	37 ± 7 (2)
		Isoxsuprine	66 ± 12 (4)	32 ± 5 (2)	37 ± 14* (2)	42 ± 4 (3)
5	16 wk	Isoxsuprine	53 ± 8 (9)	35 ± 5 (3)	47 ± 6 (6)	34 ± 6 (4)
		Placebo	66 ± 13 (4)	40 ± 12 (1)	52 ± 5 (4)	36 ± 5 (4)
6	20 wk	Isoxsuprine	58 ± 6 (6)	29 ± 9 (2)	44 ± 7 (3)	35 ± 6 (2)
		Placebo	61 ± 8 (4)	37 ± 6 (3)	53 ± 4† (3)	36 ± 6 (2)

Platelet adherence expressed as percentage of surface coverage, mean ± SD; percentage of coverage with platelet aggregates (>10 μmol/L) in parentheses. The medication and time course is marked.

*$P = .03$.

†$P = .05$.

numbers in parentheses, Table 2) which, for unexplained reasons, decreased during the course of observation. An effect of isoxsuprine on aggregation formation cannot be ruled out completely; however, this was not significant because the placebo group also showed a decrease in aggregate formation. Isoxsuprine should then also work for at least eight weeks after cessation of administration, which seems unlikely in view of the rapid disappearance of effect on the other parameters such as viscosity and platelet adherence.

Correlation of platelet adherence and RBC deformability. For perfusion conditions in which correlation was found between medication and platelet adherence (patient RBCs and donor PRP at 1,800 s^{-1}), we tested for direct correlation between platelet adherence and RBC deformability (analysis of covariance Σ adh $(3 + 4 - 5 - 6)$ *v* Σ T $(3 + 4 - 5 - 6)$. The results obtained in all patients are shown in Fig 4. The points in the graph are clearly separated into two groups, which indicates that the apparent relation between platelet adherence and RBC deformability is an effect of the medication ($P = .011$). Correlations within the groups were absent. These tests were not applied to the other type of perfusion experiments. We also tested for correlations by plotting all available values for platelet adherence *v* *T*, which yielded a set of 60 points (10 patients × 6 test points) for each type of perfusion experiment. Both variables were significantly correlated in three of four cases (recombined perfusates 1,800 s^{-1} and 300 s^{-1}, and whole blood 1,800 s^{-1}) and not significant for experiments with whole blood at wall shear rate of 300 s^{-1}. The level of significance was highest for recombined perfusates at 1,800 s^{-1}, and less for 300 s^{-1} and whole blood perfusions at 1800 s^{-1}. These data are presented in Fig. 5. Significant correlation was only found when all values (placebo and drug) were considered. Analysis of values obtained in the drug period and placebo period as separate sets yielded no correlation within these sets. To discover whether isoxsu-

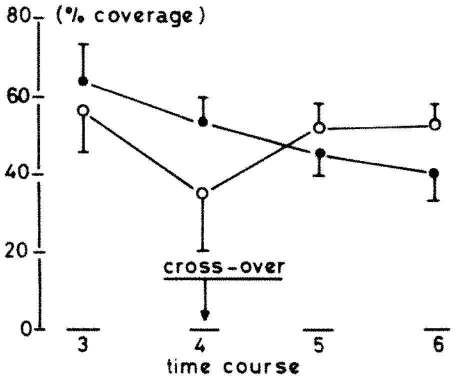

Fig 3. Platelet adherence (percentage of surface coverage, mean ± SD). A significant group-period interaction of platelet adherence with medication ($P = .017$) existed in the case of perfusion experiments with RBCs from the patients and donor platelet-rich plasma at a wall shear rate of 1,800 s^{-1} [placebo-isoxsuprine group (●); isoxsuprine-placebo group (O)]. At the separate test points, *t* tests between the two groups showed a significant difference at test point 4 ($P = .03$) and 6 ($P = .05$).

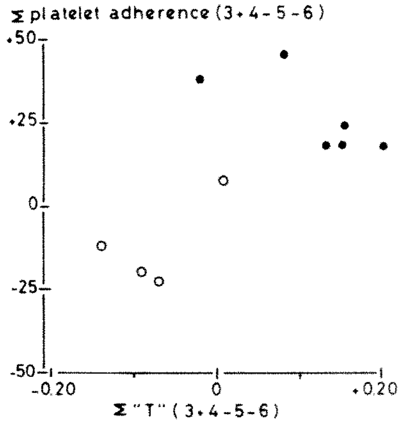

Fig 4. Correlation between platelet adherence and RBC deformability (analysis of covariance) for perfusion experiments with RBCs of the patients and donor platelet-rich plasma at a wall shear rate of 1,800 s^{-1} ($P = .011$). Placebo-isoxsuprine group (●); isoxsuprine-placebo group (O).

prine had direct influence on platelet adherence, an experiment was performed with platelets and plasma of human volunteers to whom isoxsuprine was administered for four weeks. Platelet adherence studies were performed at the beginning and end of this period. In these studies, platelets and plasma were mixed with RBCs from healthy donors, not exposed to isoxsuprine at shear rates of 300 s^{-1} and 1,800 s^{-1}; no effect of isoxsuprine could be detected (Table 3). Previous studies on in vitro treatment of RBCs with isoxsuprine have shown that the plasma concentrations achieved by in vivo administration are much too low to have an acute effect on deformability.

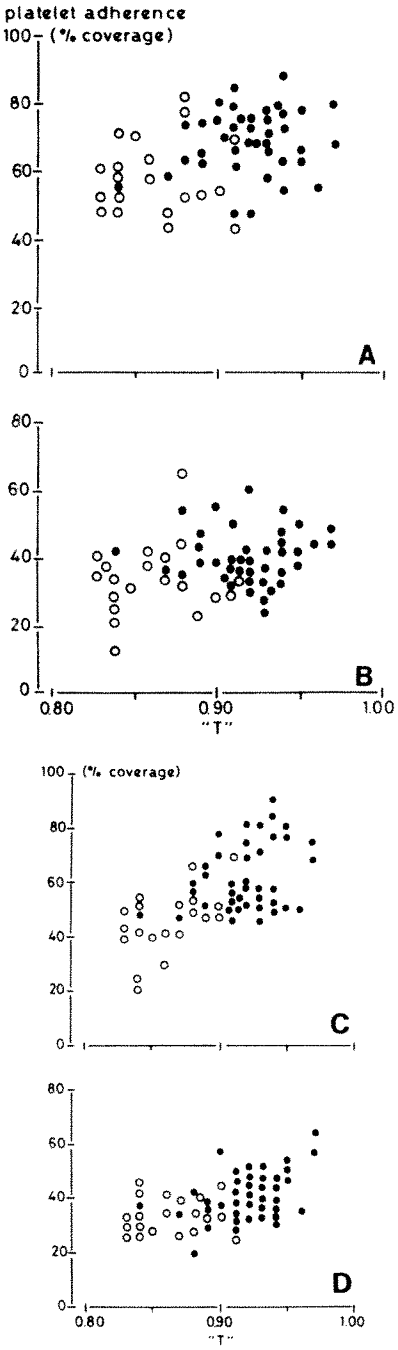

Fig 5. Overall plot obtained from all available values of platelet adherence *v* RBC deformability. Whole blood: 1,800 s^{-1} ($P < .01$) (A); 300 s^{-1} (B) (not significant). Patient red cells and donor platelet-rich plasma: 1,800 s^{-1} (C), ($P < .001$); 300 s^{-1} (D) ($P < .01$). Values obtained in the case of placebo use (●); values obtained in the case of isoxsuprine use (○).

Laboratory tests. *H*, Hb content, mcv platelet count, and leukocyte count were not influenced by medication and had values within the normal range. The values of these variables showed a random variance throughout the study.

Aggregation studies with platelets of patients performed 30 minutes after blood collections showed normal aggregability with ADP (2.5 μmol/L and 5.0 μmol/L). Plasma analysis for the platelet-specific platelet factors β-TG and platelet factor 4 showed slightly enhanced values as compared with normal values, indicating some degree of in vivo platelet activation. Levels of β-TG ranged from 50 to 90 ng/mL of plasma (normal value < 50 ng/mL of plasma). Levels of platelet factor 4 ranged from 20 to 50 ng/mL of plasma (normal value < 20 ng/mL of plasma). No treatment effect was observed on these values. Platelet aggregation studies performed with platelets of the volunteers who had received isoxsuprine for four weeks were normal and identical before and after drug therapy.

Clinical data. Of the 12 patients who entered the trial, ten completed the full course. One patient died suddenly of a ruptured aneurysm in the fourth week of the washout period; a second patient completed the first eight-week episode, in which he took isoxsuprine but refused further cooperation because of upper abdominal discomfort. No adverse effects of either isoxsuprine or placebo was reported by the remaining patients. Nine patients reported that they had benefitted from the study, possibly because of the close medical attention and the concomitant training effect on their walking behavior. Three patients enlarged with claudication distance considerably during the study period. The ankle-arm pressure indices at rest and after maximal walking distance did not improve significantly in any of the patients and, in the patient who dropped out of the study after the isoxsuprine period, a marked deterioration of these values occurred. The maximal walking distance on the treadmill improved in three patients, in two of them during the placebo period and in the third patient during the isoxsuprine period of the study.

DISCUSSION

Behavior of RBCs in flowing blood, which is determined to an appreciable extent by *H* and RBC deformability, influences blood viscosity as well as platelet transport. In a previous report, we demonstrated that RBC deformability

Table 3. Platelet Adherence in Perfusion Experiments With Platelets and Plasma From Healthy Volunteers, Mixed With RBCs From Healthy Donors, Before and After Four Weeks of Administration of Isoxsuprine

	Platelet Adherence Coverage (%)	
Shear Rate	Before	After
300 s^{-1}	44 ± 14	40 ± 11 (NS)
1,800 s^{-1}	60 ± 12	63 ± 11 (NS)

affects platelet transport towards the vessel wall and subsequent adherence.[6]

Our present study shows that isoxsuprine, when orally administered, increases RBC deformability; this had been observed previously in in vitro treatment. This increase in RBC deformability was highly significant when compared with placebo, whereas cross-over of the medication was followed by a highly significant group–period interaction in RBC deformability.

In vitro perfusion with RBCs of the patients showed a reduction of platelet adherence after use of active drug and a significant group–period interaction of this reduction at cross-over of medication. This effect, however, only occurred in perfusion experiments in which RBCs of the patients were supplemented with donor plasma and platelets at a high wall shear rate of 1,800 s^{-1}. This is consistent with the outcome of our previous in vitro study in which the influence of RBC deformability on platelet adherence was more pronoucned at a shear rate of 1,800 s^{-1} than at 300 s^{-1}. It is clear that actual cell deformation and thus differences in RBC deformability will be larger at high shear rates. Therefore, the effect of RBC deformability on platelet adherence will be larger at a shear rate of 1,800 s^{-1} than at 300 s^{-1}. An explanation for the fact that the perfusion experiments with whole blood failed to demonstrate an effect may be sought in the intraindividual and interindividual scatter of platelet count and H—two parameters which strongly influence platelet adherence. To test this, platelet adherence was normalized to the standard values of H and platelet count of 0.4 and 190,000 platelets/μL, respectively. This calculation, which is described in detail in the appendix, yielded a markedly improved correlation of platelet adherence with medication. At a shear rate of 1,800 s^{-1}, the P value for group–period interaction was 0.036. No significant effect was observed at 300 s^{-1} (Table 4). A direct enhancement of the platelet adhesiveness by the drug which could cancel out the decrease caused by the increased RBC deformability was not likely, because control perfusion experiments, in which only the platelets were in contact with the drug showed no effect (increase or decrease).

Table 4. Normalized Platelet Adherence in Whole Blood Perfusion Experiments

Test Point	Time Course	Medication	Platelet Adherence (± SD) 300 s^{-1}	1,800 s^{-1}
1	Start	Placebo	18 ± 4	41 ± 10
		Placebo	24 ± 7	45 ± 18
2	4 wk	Placebo	21 ± 11	44 ± 13
		Placebo	31 ± 10	50 ± 14
3	8 wk	Placebo	27 ± 6	39 ± 11
		Isoxsuprine	20 ± 16	36 ± 16
4	12 wk	Placebo	21 ± 6	36 ± 7
		Isoxsuprine	19 ± 6	36 ± 11
5	16 wk	Isoxsuprine	16 ± 3	26 ± 5
		Placebo	23 ± 4	43 ± 12
6	20 wk	Isoxsuprine	15 ± 4	28 ± 5
		Placebo	23 ± 4	41 ± 5

Platelet adherence was normalized to a standard hematocrit of 0.4 and a standard platelet count of 190,000/μL. An empirical relation, derived previously, which describes the dependence of platelet adherence on important parameters such as hematocrit, platelet count, and shear rate, was used for this purpose.[18] The patients' data and the empirical relationship are given in Appendix.

In the present study, correlation of RBC deformability and platelet adherence (Figs 4 and 5) could only be demonstrated when the RBC deformability was increased by isoxsuprine treatment, but not within the groups of pretreatment or posttreatment results. There are two possible explanations: first, the differences were caused exclusively by the influence of isoxsuprine on RBC deformability. Second, isoxsuprine affects both reduced deformability and platelet adherence by different independent mechanisms. The first explanation is more realistic, however, because theoretical considerations predict a close relation between RBC deformability and platelet adherence.

Marked improvement of the claudication distance was evident in three patients, and overall subjective benefit from the study period was reported by nine. It is well known, however, that close medical attention and walking training can be beneficial to patients with intermittent claudication and may lead to subjective improvement or even disappearance of intermittent claudication without objective alteration of the disease.[19,20] The amelioration noted in our patients could not be explained by improvement of ankle-arm pressure indices at rest and after maximal walking distance and appeared to be due to subjective influences, as patients' walking distances improved throughout the trial period, independent of the use of drug or placebo.

Although we did not expect a change in the disease state in this short study period, the results of the laboratory tests and perfusion experiments demonstrate a potentially important property of isoxsuprine, which is totally different from its putative vasodilating activity. Vasodilation, however, is not likely to be effective in this type of vascular diseases, as reported previously.[21,22]

We conclude that eight-week oral use of isoxsuprine results in marked decrease in RBC deformability, which in turn causes a decrease in platelet adherence in an in vitro perfusion system. The potential beneficial effect of isoxsuprine in reducing platelet adherence in the course of vascular disease will have to be established in long-term studies.

APPENDIX

Correction of platelet adherence for H and platelet count. Recently, we described an empirical relation which gives the influence of wall shear rate, H, and platelet concentration on platelet adherence in the annular perfusion chamber.[23]

$$\text{adh} = 0.83 \cdot 10^{-6} C_0 \cdot t \cdot (\gamma \cdot \gamma_0^{-1})^{0.53+0.86H-0.60H^2} \quad [2]$$

in which C_0 = platelet concentration (platelets per square centimeter), t = exposure time (s), γ_w = wall shear rate (s^{-1}) and H = hematocrit (volume fraction); γ_0 is an arbitrary shear rate of 1 s^{-1}, which is introduced to make the exponent dimensionless. The constant of 0.83 · 10^{-6} has the dimensions (cm · s^{-1}).

This relation was used to calculate the platelet adherence at the standardized H and platelet count of 0.4 and 190,000 platelets per microliter, respectively, from the real measured

Table 5. Individual Values of Hematocrit and Platelet Count of the Patients

Patients		1	2	3	4	5	6
Placebo–isoxsuprine							
1	Hematocrit	0.52	0.51	0.51	0.51	0.51	0.51
	Platelet count	330	365	417	320	333	376
2	Hematocrit	0.38	0.36	0.36	0.40	0.47	0.46
	Platelet count	376	354	396	348	308	267
3	Hematocrit	0.50	0.46	0.48	0.50	0.52	0.47
	Platelet count	321	309	314	341	330	327
4	Hematocrit	0.46	0.43	0.42	0.46	0.48	0.53
	Platelet count	196	203	224	261	216	215
5	Hematocrit	0.48	0.43	0.48	0.35	0.52	0.49
	Platelet count	275	285	286	290	328	318
6	Hematocrit	0.48	0.45	0.45	0.48	0.49	0.51
	Platelet count	338	296	250	230	319	253
Isoxsuprine–placebo							
1	Hematocrit	0.49	0.47	0.45	0.43	0.48	0.47
	Platelet count	223	218	236	216	218	223
2	Hematocrit	0.55	0.53	0.50	0.51	0.53	0.49
	Platelet count	357	360	361	285	293	307
3	Hematocrit	0.50	0.43	0.57	0.47	0.46	0.46
	Platelet count	198	209	212	215	216	224
4	Hematocrit	0.50	0.46	0.47	0.52	0.47	0.50
	Platelet count	278	302	285	307	278	290

Hematocrit in volume fraction; platelet count in 10^3 platelets per microliter.

values of adherence, *H*, and platelet count. These latter data are listed in Table 5. In eq. 2, adherence is expressed as platelets per square centimeter, whereas in our experiments, percentage of coverage is used. For conversion, a relation between platelets per square centimeter and percentage of coverage as described by Bolhuis was used[24] (1% coverage ~ 10^5 platelets per square centimeter).

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REFERENCES

1. Sixma JJ: Role of blood vessel, platelet and coagulation interactions in haemostasis, in Bloom AL, Thomas DP (eds): Haemostasis and Thrombosis. London, Churchill Livingstone, 1981, p 252

2. Mustard JP, Packham MA, Kinlough-Rathbone RL: Mechanism in thrombosis, in Bloom AL, Thomas DP (eds): Haemostasis and Thrombosis. London, Churchill Livingstone, 1981, p 503

3. Baumgartner HR, Haudenschild C: Adhesion of platelets to subendothelium. Ann NY Acad Sci USA 201:22, 1972

4. Turitto VT, Weiss HJ: Red blood cells, their dual role in thrombus formation. Science 207:541, 1980

5. Aarts PAMM, Bolhuis PA, Sakariassen KS, Heethaar RM, Sixma JJ: Red blood cell size is important for adherence of blood platelets to artery subendothelium. Blood 62:214, 1983

6. Aarts PAMM, Heethaar RM, Sixma JJ: Red blood cell deformability influences platelets–vessel wall interaction in flowing blood. Blood 64:1228, 1984

7. DiPerri T, Forconi S, Guerrine N, Agnusdei D: In vitro activity of isoxsuprine on blood, plasma and serum viscosity. Pharmatherapeutica 1:447, 1977

8. Weber G, Kreisel T, Peter S, Kunzel J: A double-blind placebo-controlled cross-over study in patients with peripheral vascular diseases using a new capillary viscometer. Angiology 31:1, 1980

9. Schlichting H, Heidrich M: Einfluss von Isoxsuprine auf die Blut Viskosität. Vasa 5:51, 1976

10. DiPerri T, Forconi S, Agnusdei D, Guerrine M, Laghi Pasini F: The effect of intravenous isoxsuprine on blood viscosity in patients with occlusive peripheral arterial disease. Br J Clin Pharmacol 5:255, 1978

11. Bruce RA: Multi-state treadmill test of submaximal and maximal exercise, in Naughton J, Hellerstein HH (eds): Exercise of Apparently Healthy Individuals: A Handbook for Physicians. New York, American Heart Association, 1972, p 37

12. Dintenfass L: Internal viscosity of the red cell and blood viscosity equation. Nature 219:956, 1968

13. Dintenfass L: Theoretical aspects and clinical applications of the blood viscosity equation containing a term for the internal viscosity of the red cell. Blood Cells 3:367, 1977

14. Sakariassen KS, Bolhuis PA, Sixma JJ: Platelet adherence to subendothelium of human arteries in pulsatile and steady flow. Thromb Res 19:547, 1980

15. Bird RB, Stewart WF, Lightfoot EN: Transport Phenomena, vol 34. New York, Wiley, 1960, p 34

16. Aarts PAMM, Van den Broek JAThM, Kuiken GDC, Sixma JJ, Heethaar RM: Velocity profiles in an annular perfusion chamber measured by Laser Doppler velocimetry. J Biomech 17:61, 1984

17. Baumgartner HR: The role of blood flow in platelet adhesion, fibrin deposition and formation of mural thrombi. Microvasc Res 5:167, 1973

18. Sato T, Shamoto M: A simple rapid polychrome stain for epoxy-embedded tissue. Stain Technol 48:223, 1973

19. Dahlhof AG, Holm J, Schersten T. Exercise training of patients with intermittent claudication. Scand J Rehab Med 9:20, 1983 (suppl)

20. Clifford PC, Davies PW, Hayne JH, Baird RW: Intermittent claudication: Is a supervised exercise worthwhile. Med J 208:1503, 1980

21. Strandness DE: Ineffectiveness of isoxsuprine on intermittent claudication. JAMA 213:86, 1972

22. Coffman JD, Mannich JH: Failure of vasodilator drugs in arteriosclerosis obliterans. Ann Intern Med 76:35, 1972

23. Aarts PAMM, Steendijk P, Sixma JJ, Heethaar RM: Shear-dependent red cell motion as a possible mechanism for platelet diffusivity in flowing blood. J Biomech (in press)

24. Bolhuis PA, Sakariassen KS, Sander HJ, Bouma BN, Sixma JJ: Binding of factor VIII–von Willebrand factor to subendothelium precedes increased platelet adherence and enhances platelet spreading. J Lab Clin Med 97:568, 1981

A Monoclonal Antibody Preventing Binding of Tissue-Type Plasminogen Activator to Fibrin: Useful to Monitor Fibrinogen Breakdown During t-PA Infusion

By P. Holvoet, H.R. Lijnen, and D. Collen

One (MA-1C8) of 36 monoclonal antibodies obtained by fusion of P3X63-Ag8-6.5.3 myeloma cells with spleen cells of mice immunized with purified human tissue-type plasminogen activator (t-PA) blocked the activity of t-PA on fibrin plates but not on chromogenic substrates. MA-1C8 at a concentration of 200 μg/mL inhibited plasma clot lysis and binding of t-PA to the clot. MA-1C8 had no influence on the activation of plasminogen by t-PA, which obeys Michaelis-Menten kinetics with $K_m = 105$ μmol/L and $k_{cat} = 0.05$ s^{-1}; however, it abolished the influence of CNBr-digested fibrinogen on K_m. These findings confirm that the stimulatory effect of fibrin on the activation of plasminogen by t-PA is mediated by binding of t-PA to fibrin and provide additional support for the kinetic model. Addition of t-PA to pooled fresh human plasma to a concentration of 5 μg/mL resulted in extensive fibrinogen breakdown after incubation for one hour at 37 °C or during storage at −20 °C for one day. In both instances, fibrinogen degradation was completely prevented by addition of MA-1C8 to a concentration of 200 μg/mL of plasma. MA-1C8 also effectively prevented in vitro fibrinogen degradation and in vitro plasminogen activation in plasma samples obtained during infusion of recombinant t-PA in patients with thromboembolic disease. Thus, MA-1C8 is a useful tool for discriminating between in vivo and in vitro fibrinolysis during thrombolytic therapy with t-PA.

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TISSUE-TYPE plasminogen activator (t-PA) is a single-chain glycoprotein with a mol wt of $\sim$70,000, that converts the proenzyme plasminogen to the fibrinolytic enzyme plasmin. The activation of plasminogen by t-PA obeys Michaelis-Menten kinetics with a K_m of 65 μmol/L in the absence of fibrin but with a K_m of 0.16 μmol/L in the presence of fibrin.[1] Kinetic analysis indicated that the effect of fibrin on the activation rate of plasminogen is due to binding of t-PA to fibrin followed by high-affinity addition of plasminogen to the t-PA-fibrin complex.[1]

Here, we describe a t-PA–specific monoclonal antibody (MA-1C8) that prevents the binding of t-PA to fibrin and thereby abolishes the effect of fibrin on the activation of plasminogen by t-PA. This antibody also appears to prevent effectively the activation of the fibrinolytic system in human plasma by high concentrations of t-PA.

MATERIALS AND METHODS

Purified proteins. Human t-PA was isolated from the culture fluid of human melanoma cells.[2] The activity was expressed in international units by comparison of the fibrinolytic activity in plasminogen-enriched bovine fibrin films[3] with that of the 1st International Reference Preparation of t-PA (83/517), which was obtained from Dr P.J. Gaffney, National Institute of Biological Standards and Control, London. The protein concentration of t-PA was measured by amino acid analysis as detailed by Rijken and Collen.[2] In this way, a specific activity of $\sim$500,000 IU/mg was obtained for purified t-PA.

Glu-plasminogen was prepared from human plasma by chromatography on lysine-sepharose followed by gel filtration.[4] Its concentration was determined spectrophotometrically using the absorbance coefficient $A^{280nm/1cm} = 16.8$.[5] The plasminogen was >95% activatable as measured by active site titration using *p*-nitrophenyl-*p*′-guanidinobenzoate[6] after activation with streptokinase (1,500 IU/mg of protein) in 0.1 mol/L phosphate buffer, pH 7.30, containing 25% glycerol at 0 °C for two hours.

Fibrinogen was prepared from fresh frozen blood bank plasma according to the method of Blombäck and Blombäck[7]; its concentration was determined spectrophotometrically using $A^{280nm/1cm} = 15.1$. Fibrinogen was dissolved in 70% formic acid to a protein concentration of 10 mg/mL and digested with CNBr (100-fold molar excess over methionine) for 17 hours at room temperature under a nitrogen atmosphere. The resulting CNBr-fragments were dialyzed extensively against distilled water and stored frozen until used. Complete digestion of fibrinogen was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Reagents. Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Streptokinase was obtained from Kabi (Stockholm) and the chromogenic substrates D-Val-Leu-Lys-*p*-nitroanilide (S-2251) and D-Ile-Pro-Arg-*p*-nitroanilide (S-2288) were purchased from Kabi-Vitrum (Amsterdam). The synthetic inhibitor D-Ile-Pro-Arg-CH_2Cl was obtained by custom synthesis at UCB (Brussels). Thrombin (Topostasine) from Roche (Basel, Switzerland) was used. Horseradish peroxidase (HRP) (275 IU/mg) was from Sigma Chemical Company (St Louis). Bovine serum albumin (BSA) (Boseral) was from Organon Technika (Oss, The Netherlands). Tween-80 was obtained from Baker Chemicals (Deventer, The Netherlands).

Fibrinogen assay. Coagulable fibrinogen was determined with the fibrin polymerization time (FPT) assay.[8]

Immunization of Balb/c mice. Balb/c mice were primed subcutaneously and intraperitoneally with 50 μg of purified t-PA dissolved in 0.2 mL of saline, which was mixed with 0.1 mL of complete Freund's adjuvant. After two weeks, the mice were further immunized by intraperitoneal injection of 50 μg of the antigen in saline and incomplete Freund's adjuvant. On the fourth and second days before fusion, mice were boosted intraperitoneally with the same amount of antigen in saline.

Cell hybridization. P3X63-Ag8-6.5.3 myeloma cells[9] were fused with spleen cells from immunized Balb/c mice in the presence of polyethylene glycol 4000 (Merck, Darmstadt, F.R.G.) according to the Fazekas and Scheidegger[10] modification of the Köhler and Milstein hybridization techniques.[11] The myeloma cells were obtained through the courtesy of Dr O. Schönherr, Organon.

The supernatants were screened for specific antibody production using a micro-enzyme-linked immunosorbent assay (ELISA)

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described below. The positive clones were subcloned by limiting dilution. Large quantities of the monoclonal antibodies were obtained from ascites fluid produced in Balb/c mice.

Isotype determination and purification of monoclonal antibodies. The subclass of the monoclonal antibodies were identified using rabbit antisera against mouse IgM(Fc), IgG(Fc), IgG_1, IgG_{2b}, IgG_3, κ-chain and λ-chain and goat antisera against mouse IgG_{2a} (Nordic, Tilburg, The Netherlands).

The IgG fractions of monoclonal antibodies were purified on protein A-Sepharose[12]; 400 μL of ascites fluid was applied to a 0.9 × 15 cm column. The column was washed with 30 mL of 0.1 mol/L of Tris/HCl, pH 8.1. Elution was performed with 0.1 mol/L of glycine/HCl, pH 2.9. Fractions of 2 mL were collected, and the pH was neutralized with Tris/HCl, pH 9.0. The column was developed at a flow rate of 12 mL/h at 4 °C.

One-site, noncompetitive ELISA for detection of specific antibody production. ELISA was performed according to Engvall and Perlman[13] as detailed by Voller et al.[14] Wells of micro-ELISA plates (Titertek, Flow, Irvine, Scotland) were incubated with 200 μL of a solution of t-PA (4 μg/mL) in coating buffer, overnight at 4 °C, and treated with phosphate-buffered saline (PBS) containing 1% BSA. The wells were then washed three times with PBS containing 0.002% Tween-80 (PBS-Tween), incubated for two hours with 180 μL of conditioned culture fluid or ascites fluid in appropriate dilutions and washed again three times with PBS-Tween. The wells were then incubated for one hour with HRP-conjugated rabbit IgG raised against mouse immunoglobulins (Nordic) and washed three times for five minutes with PBS-Tween. The peroxidase reaction was then performed by addition of 100 μg/mL *o*-phenylene-diamine (Fluka, Buchs, Switzerland) and 0.003% hydrogen peroxide in 0.1 mol/L of citrate, 0.2 mol/L of sodium phosphate buffer pH 5.0. After one hour, the reaction was arrested with 50 μL of 4 mol/L of sulphuric acid, and the absorbance was read at 492 nm in a Titertek Multiskan (Flow).

Distinction of epitopes. The monoclonal antibodies are divided into groups specific for different epitopes according to Stähli et al.[15]

Measurement of concentration of t-PA by a two-site ELISA. Wells of micro-ELISA plates were coated with the IgG fraction (4 μg/mL) of rabbit antiserum raised against human t-PA. After treatment with PBS-BSA, 180-μL samples were applied to the wells and incubated for 16 hours at 4 °C. The wells were washed with PBS-Tween and incubated for two hours with diluted HRP-conjugated monoclonal anti-t-PA antibody (MA-62E8).[16] The peroxidase reaction was performed as described above. Standard curves were constructed by dilution of human melanoma t-PA (final concentration between 20 and 0.15 ng/mL) in PBS-Tween-BSA.

Quenching of fibrinolytic activity of t-PA on fibrin plates. The fibrinolytic activity of t-PA (2, 1, 0.5 μg/mL) in the absence and the presence of t-PA specific antibodies was determined by applying 10-μL aliquots to plasminogen-containing bovine fibrin plates.[3] In the quenching experiments, monoclonal antibodies were used in a 40-fold molar excess. The mean residual fibrinolytic activity was calculated from a standard curve (t-PA solutions containing between 0.02 and 20 μg/mL).

Binding of t-PA to plasma clots and subsequent clot lysis. Samples of monoclonal antibody MA-1C8 (final concentration between 0 and 25 μL) were preincubated with t-PA (final concentration 0.14 μmol/L) for one hour at 37 °C. To 100 μL of this preincubation mixture, 900 μL of normal human plasma was added and the mixture was divided in two 500-μl aliquots which were clotted by addition of thrombin (final concentration 8 IU/mL of plasma). To one aliquot, the synthetic t-PA inhibitor D-Ile-Pro-Arg-CH_2Cl[17] (final concentration 10^{-5} mol/L) was added immediately after clotting. After centrifugation at 4,000 g, the t-PA concentration in the clot supernatants was determined with the ELISA as described above. The other aliquot was used to measure the clot lysis time. A calibration curve was obtained by measuring the lysis time of plasma clots produced in the presence of t-PA (final concentration between 0.15 and 5 μg/mL plasma) in the absence of monoclonal antibody. In control experiments, t-PA was preincubated with monoclonal antibody MA-25G5.

Quantitation of plasmin-α_2-antiplasmin (PAP) complex in human plasma. PAP in human plasma was measured with a sandwich-type micro-ELISA assay.[18] Therefore, microtiter plates were coated with monoclonal antibodies against α_2-antiplasmin, and bound antigen was quantitated with HRP-conjugated monoclonal antibody reacting equally well with Glu-plasmin(ogen) as with Lys-plasmin(ogen).

Influence of MA-1C8 on the kinetics of plasminogen activation by t-PA. Glu-plasminogen (final concentration 10 to 100 μmol/L) was incubated at 37 °C with t-PA (final concentration 2 μg/mL) in 0.01 mol/L of Na_2HPO_4, 0.14 mol/L of NaCl pH 7.40, containing 0.25% gelatin, 0.01% Tween-80, and 0.1% sodium azide. The generated plasmin was measured spectrophotometrically at 405 nm at different time intervals (0 to 10 minutes) using S-2251 (final concentration 0.3 mmol/L) after the sample was diluted 50-fold to stop the activation reaction. At these final substrate and enzyme concentrations, the activation rate is negligible during the measuring period. Furthermore, under these conditions, the amount of generated plasmin is small as compared with the concentration of plasminogen and conversion of Glu-plasminogen to Lys-plasminogen is not observed. Initial activation rates were obtained from plots of generated plasmin concentration *v* activation time.

Alternatively, Glu-plasminogen (final concentration 0.12 to 0.75 μmol/L) was incubated at 37 °C in PBS pH 7.40, with different amounts of CNBr-digested fibrinogen (CNBr-Fg) (final concentration 0.27 to 3.3 μmol/L) prior to addition of t-PA (final concentration 2 μg/mL). Activation of Glu-plasminogen (final concentration 10 to 100 μmol/L) by t-PA (2 μg/mL final concentration) preincubated with a fourfold or higher molar excess of $F(ab')_2$ fragments from MA-1C8 was measured both in the presence and the absence of CNBr-Fg. The results were analyzed by linear regression.

Plasma samples from patients during thrombolytic therapy with t-PA. Blood was collected from 11 patients with thromboembolic disease (deep vein thrombosis, pulmonary embolism, or acute myocardial infarction) treated by intravenous (IV) infusion of recombinant human t-PA (40 mg over 90 minutes).[19,20] Blood was collected both on citrate (final concentration 0.01 mol/L) and on citrate and MA-1C8 (final concentration 200 μg/mL), before the start of the infusion, at 30-minute intervals during the infusion and at 30 minutes after the end of the infusion. After centrifugation at 4,000 g, the plasma samples were immediately frozen, stored at −20 °C, and individually thawed for immediate fibrinogen assay. The concentration of t-PA in the plasma samples was quantitated in a micro-ELISA based on monoclonal antibodies.[21]

Other techniques. Immunodiffusion was performed according to Ouchterlony.[22] Amino acid analysis was performed on a Beckman 119 CL amino acid analyzer after hydrolysis in 6 mol/L of HCl in vacuo at 110 °C for 20 hours. $F(ab')_2$ fragments of monoclonal antibody 1C8 were obtained after pepsin digestion.

RESULTS

Identification of anti-t-PA monoclonal antibodies. Supernates of hybridomas resulting from fusions of P3X63-Ag8-6.5.3 myeloma cells with spleen cells of mice immunized with purified t-PA were screened for specific antibody production using the one-site micro-ELISA described above.

Nine fusions yielded 36 hybridomas producing monoclonal antibodies reacting with 25 different epitopes in the t-PA molecule.[15] The subclass of the heavy chains was determined by immunodiffusion. Two anti-t-PA monoclonal antibodies (MA-M2C6, MA-24G10) appeared to be IgM, and all the others appeared to be IgG_1. All antibodies have a κ-light chain.

Quenching of fibrinolytic activity of t-PA on fibrin plates. The fibrinolytic activity of samples containing 2, 1, and 0.5 μg/mL of t-PA was measured on fibrin plates before and after incubation with a 40-fold molar excess of 25 different t-PA specific IgG_1 monoclonal antibodies. The activity was not influenced in the presence of 20 of the monoclonal antibodies. After incubation with three monoclonal antibodies (MA-62E8, MA-3B6, and MA-1D10), a residual t-PA activity of 40% was found. Monoclonal antibodies MA-1C8 and MA2G6 inhibited the activation of plasminogen in the presence of fibrin almost completely (5% to 10% residual activity). MA-2G6 quenched the amidolytic activity of t-PA on the synthetic substrate S-2288, whereas MA-1C8 did not. In the present study, MA-1C8 was further investigated.

Plasma clot lysis and binding of t-PA to plasma clots. Figure 1 illustrates the effect of increasing concentrations of MA-1C8 on the binding of t-PA to plasma clots. A two-site micro-ELISA was used to measure the concentration of t-PA in the supernatants after clotting with thrombin (8 IU/mL) and centrifugation at 4,000 g. In the absence of MA-1C8, 75% of the t-PA was bound to the clots. In the presence of 2 μmol/L of of MA-1C8, the fraction of bound t-PA decreased to 55% percent; in the presence of 16 μmol/L, 85% of the t-PA was recovered in the supernatant. Preincubation of t-PA with MA-25G5 did not influence the binding of t-PA to the clots.

In the presence of 2 μmol/L of MA-1C8, the t-PA activity read on the clot lysis calibration curve was decreased to 35%. In the presence of 16 μmol/L, the residual t-PA activity was reduced to 10%.

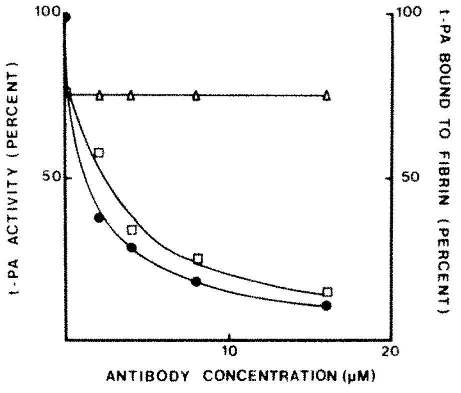

Fig 1. Plasma clot lysis and binding of t-PA to plasma clots. Percentage of tissue-type plasminogen activator (t-PA) bound to fibrin in plasma clots after incubation of t-PA with MA-1C8 (□) or with MA-25G5 (△) is measured in micro-enzyme-linked immunosorbent assay. Residual binding is plotted *v* concentration of monoclonal antibody in the preincubation mixture; t-PA activity (percentage) in clot lysis after preincubation of t-PA with MA-1C8 (●) is plotted *v* concentration of monoclonal antibody.

Influence of MA-1C8 on the kinetics of plasminogen activation by t-PA in the presence and absence of CNBr-Fg. Activation of plasminogen by t-PA before and after preincubation with either intact MA-1C8 or $F(ab')_2$ fragments in a pure system obeyed Michaelis-Menten kinetics, as evidenced by linear plots of the inverse of the initial activation rate (1/v) *v* the inverse of the plasminogen concentration (1/[P]). The kinetic constants were 102 μmol/L for K_m and 0.034 s^{-1} for k_{cat}, which were very similar to those previously reported.[1,23,24] Preincubation of t-PA with $F(ab')_2$ fragments from MA-1C8 (four- to 40-fold molar excess) did not influence the kinetic parameters for the activation of plasminogen (Table 1).

Addition of CNBr-Fg resulted in a concentration-dependent increase of the activation rate of Glu-plasminogen by t-PA. The kinetic parameters obtained were 0.28 μmol/L for K_m and 0.13 s^{-1} for k_{cat}, corresponding to an ~1,500-fold increase in catalytic efficiency (k_{cat}/K_m) in the presence of *CNBr*-Fg.

Preincubation of t-PA with a fourfold molar excess of MA-1C8 counteracted the stimulating effect of CNBr-Fg; the kinetic constants obtained for the activation of plasminogen in the presence of saturating concentrations of CNBr-Fg (3.3 μmol/L) were 143 μmol/L for K_m and 0.12 s^{-1} for k_{cat}. A further increase of the concentration of MA-1C8 (up to 40-fold molar excess) did not influence the kinetic parameters (Table 1).

Inhibition of in vitro degradation of fibrinogen in t-PA-enriched human plasma. To pooled human plasma samples, t-PA (final concentration between 0.15 and 5 μg/mL) were added. The samples were incubated at 37 °C for 20, 60, and 240 minutes, and the fibrinogen concentration was measured. After 240 minutes, extensive fibrinogen degradation is observed at concentrations of t-PA above 1.25 μg/mL (Table 2). When plasma samples enriched with t-PA were frozen immediately and stored at − 20 °C for 1, 7, or 30 days, a comparable extensive degree of fibrinogen breakdown was observed at concentrations above 1.25 μg/mL (Table 2). When t-PA was added to plasma containing

Table 1. Kinetic Parameters for the Activation of Glu-Plasminogen by t-PA Before and After Preincubation With MA-1C8 in the Presence and in the Absence of CNBr-Digested Fibrinogen

	K_m (μmol/L)	k_{cat} (s^{-1})	k_{cat}/K_m (μmol/L^{-1}.s^{-1})	*r* Value
Without CNBR-Fg				
t-PA	102	0.034	0.0003	.997
t-PA with 40-fold excess of MA-1C8	105	0.062	0.0006	.999
With CNBr-Fg				
t-PA	0.28	0.13	0.464	.994
t-PA with 40-fold excess of MA-1C8	143	0.12	0.0008	.999

t-PA, tissue-type plasminogen activator.

Correlation coefficient (*r*) obtained by linear regression analysis.

Table 2. In Vitro Degradation of Fibrinogen by t-PA in Human Plasma During Incubation at 37 °C or Storage at −20 °C

	Incubation at 37 °C			Storage at −20 °C		
t-PA (µg/mL)	20 min	60 min	240 min	1 day	7 days	30 days
0.15	100	100	100	100	94	80
0.31	100	100	100	85	84	75
0.62	100	100	90	80	76	60
1.25	95	90	75	65	62	40
2.50	95	90	35	20	20	15
5.00	90	35	20	10	10	7
5 + 200 µg/mL MA-1C8	100	100	100	100	100	100

Values indicate the residual fibrinogen concentrations expressed in percentages.

monoclonal antibody MA-1C8 at a concentration of 200 µg/mL, the fibrinogen breakdown during incubation at 37 °C or during storage at −20 °C, was completely abolished.

Discrimination between in vivo and in vitro degradation of fibrinogen in plasma of patients during thrombolytic therapy with t-PA. Blood samples from 11 patients during and after thrombolytic therapy with recombinant t-PA were collected on citrate in the presence and in the absence of monoclonal antibody MA-1C8 (final concentration 200 µg/mL). The mean residual fibrinogen concentration in the samples without monoclonal antibodies decreased to 73% ± 12% (mean ± SD) of the preinfusion level after 30 minutes and to 41% ± 24% after 90 minutes. Thirty minutes after the end of the infusion (t = 120 minutes), the apparent fibrinogen concentration was increased to 69% ± 29% (Fig 2).

In plasma collected on monoclonal antibody MA-1C8 (final concentration 200 µg/mL), the apparent fibrinogen breakdown was far less pronounced. The residual fibrinogen levels were 94% ± 6% after 30 minutes, 77% ± 22% after 90 minutes, and 74% ± 20% after 120 minutes (Fig 2).

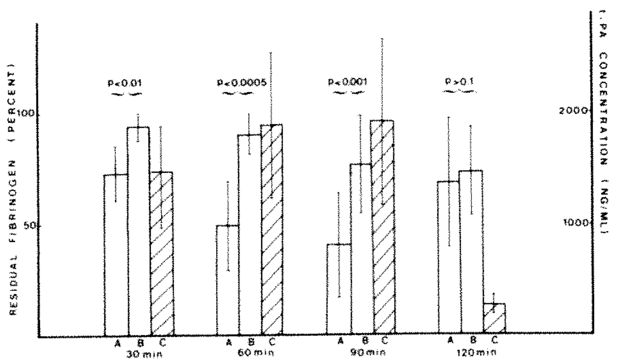

Fig 2. Degradation of fibrinogen during thrombolytic therapy with tissue-type plasminogen activator (t-PA). Blood samples of patients (n = 11) were collected in citrate (0.1 vol of 0.11 mol/L) in the absence (A) and in the presence (B) of MA-1C8 (final concentration 200 µg/mL). The fibrinogen concentration as determined in a coagulation rate assay, is expressed in percentage of the preinfusion level. The concentration of t-PA antigen (ng/mL) is determined by enzyme-linked immunosorbent assay (C). All patients received 40 mg of t-PA over 90 minutes.

Discrimination between in vivo and in vitro plasminogen activation. Blood samples from 11 patients during and after thrombolytic therapy with recombinant t-PA were collected on citrate in the presence and in the absence of monoclonal antibody MA-1C8. The mean PAP concentration in the samples without monoclonal antibody increased to 490 ± 110 nmol/L after 60 minutes and to 540 ± 100 nmol/L after 90 minutes. Thirty minutes after the end of the infusion (t = 120 minutes), the mean PAP concentration was decreased to 210 ± 50 nmol/L.

In plasma samples collected on monoclonal antibody MA-1C8 (final concentration 200 µg/mL), the apparent plasminogen activation was far less pronounced. The levels of PAP were 160 ± 30 nmol/L after 60 minutes, 250 ± 60 nmol/L at 90 minutes and 180 ± 60 nmol/L 30 minutes after the end of infusion (Fig 3).

DISCUSSION

Of 36 t-PA specific monoclonal antibodies, only two (MA-2G6 and MA-1C8) quenched the fibrinolytic activity of tPA on fibrin plates. Although MA-2G6 also inhibited the amidolytic activity of t-PA on the synthetic substrate S-2288, MA-1C8 did not. This suggests that MA-1C8 is not directed against an epitope near the active site of t-PA, whereas MA-2G6 could be directed against the active site or could change the structure of the active site after binding. MA-1C8 is further investigated in the present study.

The influence of MA-1C8 on the kinetics of the activation of Glu-plasminogen by t-PA was studied. Several groups have shown a stimulatory effect of fibrin on the activation rate of plasminogen by t-PA. Hoylaerts et al[1] reported a dramatic decrease of the K_m (from 65 µmol/L to 0.16 µmol/L in the presence of fibrin) and only a minor change of k_{cat} (0.06 to 0.1 s^{-1}). Similar results were obtained by Zamarron et al[23] using CNBr-Fg as a soluble cofactor. Rånby[24] found, however, that both K_m and k_{cat} change when

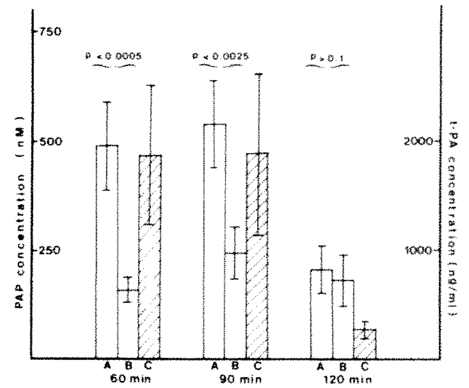

Fig 3. Plasminogen activation during thrombolytic therapy with tissue-type plasminogen activator (t-PA). Blood samples of patients (n = 11) were collected in citrate (0.1 vol of 0.11 mol/L) in the absence (A) and presence (B) of MA-1C8 (final concentration 200 µg/mL). The concentration of PAP as measured in micro-enzyme-linked immunosorbent assay (ELISA) is expressed in nmol/L. The concentration of t-PA antigen (ng/mL) is determined by ELISA (C).

fibrin is added (tenfold decrease of K_m and 15-fold increase of k_{cat}). Although Nieuwenhuizen et al[25] obtained a lower K_m for the activation in the absence of fibrin (0.053 μmol/L), they also found a change in both K_m (0.003 μmol/L) and k_{cat} (30-fold increase).

Preincubation of t-PA with a four- to 40-fold molar excess of MA-1C8 resulted in an increase of the K_m for the activation of Glu-plasminogen in the presence of CNBr-Fg. The values for K_m in the presence and absence of the soluble cofactor were found to be similar (143 μmol/L and 105 μmol/L, respectively). MA-1C8 thus completely quenched the stimulatory effect of the fibrinogen fragments.

After preincubation of t-PA with a 40-fold molar excess of MA-1C8, the binding of t-PA to clots generated in normal human plasma was completely inhibited, and the fibrinolytic activity of t-PA was strongly reduced.

Our finding that MA-1C8 inhibits the binding of t-PA to fibrin and results in a strong increase of K_m in the presence of CNBr-Fg to a value comparable to that obtained in its absence provides additional support for the mechanism of the stimulatory effect of fibrin on the activation of plasminogen by t-PA, which was based on kinetic analysis.[1]

Incubation of plasma samples with very high concentrations of t-PA (up to 5 μg/mL) at 37 °C for one hour resulted in a 65% decrease of fibrinogen levels as measured with a clotting rate assay. A similar in vitro breakdown of fibrinogen was observed in plasma samples stored at −20 °C for upto 30 days. This in vitro degradation of fibrinogen could, however, be completely abolished when t-PA was added to plasma containing 200 μg/mL of MA-1C8.

In plasma samples obtained from patients (n = 11) during infusion of 40 mg t-PA over 90 minutes, a significant decrease of the fibrinogen concentration (to 41% ± 24% at the end of infusion) was measured. Thirty minutes later, the apparent fibrinogen concentration was again increased to 69% ± 29%. In plasma samples obtained from blood collected on MA-1C8 (final concentration 200 μg/mL), the fibrinogen concentration decreased only to 77% ± 22%, a value that is comparable to that obtained 30 minutes later, when the plasma t-PA level was markedly lower.

The decrease of fibrinogen to ~70% of the preinfusion value, measured in blood samples collected on the monoclonal antibody at the end of the t-PA infusion as well as in samples collected without antibody but 30 minutes after the end of the infusion (when the plasma level of rt-PA has decreased about tenfold), most likely represents genuine fibrinogen breakdown occurring in vivo during rt-PA infusion. The additional decrease of fibrinogen to ~50% of the preinfusion value measured in plasma samples collected in the absence of antibody at the end of the rt-PA infusion (when its concentration in plasma is high) represents fibrinogen breakdown after collection of the blood sample.

The concentrations of PAP measured in plasma samples from 11 patients, collected during thrombolytic therapy in the presence of MA-1C8 (final concentration 200 μg/mL) were significantly lower than those obtained with samples collected in its absence. Thirty minutes after the end of infusion, when the plasma rt-PA level had decreased significantly (half-life t½ = seven minutes),[19] the values in both series of samples were comparable.

From these experiments, it is concluded that the observed differences in fibrinogen levels and the extent of plasminogen activation in plasma samples obtained in the presence and in the absence of MA-1C8 during thrombolytic therapy with t-PA can be accounted for by fibrinolytic activation and in vitro breakdown of fibrinogen after collection of the blood sample. This in vitro degradation represents an artifact which prevents accurate quantitation of the in vivo systemic fibrinolytic activation and fibrinogen breakdown but which can be prevented with MA-1C8.

REFERENCES

1. Hoylaerts M, Rijken DC, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. J Biol Chem 257:2912, 1982

2. Rijken DC, Collen D: Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 256:7035, 1981

3. Astrup T, Müllertz S: The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 40:346, 1952

4. Deutsch DG, Mertz ET: Plasminogen: Purification from human plasma by affinity chromatography. Science 170:1095, 1970

5. Nilsson T, Sjöholm I, Wiman B: Circular dichroism studies on α_2-antiplasmin and its interactions with plasmin and plasminogen. Biochim Biophys Acta 705:264, 1982

6. Chase T Jr, Shaw E: Titration of trypsin, plasmin and thrombin with *p*-nitrophenyl *p*-guanidinobenzoate HCl. Methods Enzymol 19:20, 1970

7. Blombäck B, Blombäck M: Purification of human and bovine fibrinogen. Ark Kemi 10:415, 1956

8. Vermylen C, De Vreker RA, Verstraete M: A rapid enzymatic method for assay of fibrinogen: The fibrin polymerization test (F.P.T.). Clin Chim Acta 8:418, 1963

9. Kearney JF, Radbruch A, Liesegang B, Rajewski K: A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol 123:1548, 1979

10. Fazekas de St. Groth S, Scheidegger D: Production of monoclonal antibodies: Strategy and tactics. J Immunol Methods 35:1, 1980

11. Köhler G, Milstein C: Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur J Immunol 6:511, 1976

12. Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Immunochemistry 15:429, 1978

13. Engvall E, Perlman P: Enzyme-linked immunosorbent assay ELISA. III. Quantitation of specific antibodies by enzyme labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 109:129, 1972

14. Voller A, Bidwell DE, Barlett A: Microplate enzyme immunoassays for the immunodiagnosis of virus infections, in Rose NR, Friedman H (eds): Manual of Clinical Immunology. Washington, American Society of Microbiology, 1976, p 506

15. Stähli C, Miggiano V, Stocker J, Staehelin T, Häring P, Takacs B: Distinction of epitopes by monoclonal antibodies. Methods Enzymol 92:242, 1983

16. Nakane PK, Kawaoi A: Peroxidase-labeled antibody. A new method for conjugation. J Histochem Cytochem 22:1084, 1974

17. Lijnen HR, Uytterhoeven M, Collen D: Inhibition of trypsin-like serine proteinases by tripeptide arginyl and lysyl chloromethylketones. Thromb Res 34:431, 1984

18. Holvoet P, Lijnen HR, Collen D: A monoclonal antibody specific for Lys-plasminogen. Application to the study of the activation pathways of plasminogen in vivo. J Biol Chem 260:12106, 1985

19. Verstraete M, Bounameaux H, De Cock F, Van de Werf F, Collen D: Pharmacokinetics and systemic fibrinogenolytic effects of recombinant human tissue-type plasminogen activator (rt-PA) in man. J Pharm Exp Ther 235:506, 1985

20. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D: Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. Nature 301:214, 1983

21. Holvoet P, Cleemput H, Collen D: Assay of human tissue-type plasminogen activator (t-PA) with an enzyme-linked immunosorbent assay (ELISA) based on three murine monoclonal antibodies to t-PA. Thromb Haemost 54:684, 1985

22. Ouchterlony O: Diffusion-in-gel methods for immunological analysis, in Kallos P (ed): Progress in Allergy, vol V. Basel, Karger, 1958, p 1

23. Zamarron C, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by natural and recombinant tissue-type plasminogen activator. J Biol Chem 259:2080, 1983

24. Rånby M: Studies on the kinetics of plasminogen activation by tissue plasminogen activator. Biochim Biophys Acta 704:461, 1982

25. Nieuwenhuizen W, Voskuylen M, Traas DW, Hoegee-de-Nobel B, Verheijen J: Kinetics of the tissue-type plasminogen activator-mediated activation of plasminogen. Influence of CNBr-fibrin(ogen) fragment FCB-2 and different forms of plasminogen, in Henschen A, Hessel B, McDonagh J, Saldeen T (eds): Fibrinogen, Structure and Function. Berlin, de Gruyter, 1985, vol 3, p 331.

Interaction of Factor XIa and Antithrombin in the Presence and Absence of Heparin

By David L. Beeler, James A. Marcum, Sandra Schiffman, and Robert D. Rosenberg

We have studied the interaction between purified human factor XIa and antithrombin in the presence and absence of well-characterized preparations of heparin. The concentrations of hemostatic enzyme, protease inhibitor, and mucopolysaccharide were 5.76×10^{-8} mol/L, 5.76×10^{-6} mol/L, and either 5.88×10^{-6} mol/L or 0, respectively. Kinetic investigation of this process using a tritiated factor IX substrate demonstrated that the pseudo first-order rate constants of this reaction in the presence and absence of heparin are ~ 1.0 min^{-1} and ~ 0.025 min^{-1}, respectively. Thus, the rate of hemostatic enzyme–protease inhibitor complex formation is accelerated by about 40-fold in the presence of saturating levels of the mucopolysaccharide. These results were confirmed in a qualitative manner by directly monitoring the generation of factor XIa–antithrombin interaction product with sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using an antibody population specific for the protease inhibitor.

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THE PROTEASE inhibitor, antithrombin, is present in human plasma at an average concentration of 150 μg/mL.[1,2] The protease inhibitor neutralizes the activity of thrombin by formation of a 1:1 stoichiometric complex between enzyme and inhibitor through reactive site (arginine)–active center (serine) interactions.[1,3] In the absence of heparin, complex formation occurs at a relatively slow rate. When heparin is present, the mucopolysaccharide binds to lysyl residues on antithrombin and dramatically accelerates the rate of complex formation.[3,4] We surmised that the heparin-induced acceleratory phenomenon was predominantly due to an allosteric alteration in the position of the critical arginine residue or of a secondary enzyme binding site such that the protease inhibitor was more readily available for interaction with thrombin.[4,5]

The coagulation cascade is composed of a series of linked proteolytic reactions. Work from our laboratory and that of other groups has shown that coagulation factors IIa, IXa, Xa, XIa, and XIIa are inactivated by antithrombin in a manner similar to that described above.[6-16] Furthermore, heparin is able to accelerate each of these coagulation enzyme–protease inhibitor interactions dramatically. Detailed kinetic examinations of the interactions of factors IIa, IXa, and Xa with antithrombin and heparin have been published.[17,18] However, similar studies with factors XIa and XIIa have not been reported, although Damus et al,[7] Stead et al[8] Fujikawa et al,[10] and Kurachi and Davie[11] provided qualitative evidence indicating that heparin facilitates the inactivation of these hemostatic enzymes by antithrombin. Recently, Scott et al[19] claimed that the factor XIa–antithrombin interaction is minimally accelerated by the addition of heparin and that this kinetic phenomenon is of no significance in the blood. In this communication, we show that the mucopolysaccharide plays a significant role in the enhancement of factor XIa neutralization by the protease inhibitor and suggest that this effect may be of considerable physiologic importance.

MATERIALS AND METHODS

Proteins. Factors IX and XI and antithrombin were prepared from human plasma in physically homogenous form by techniques outlined in previous reports from our laboratories.[17,20] Human factor XI was activated by the addition of purified bovine pancreatic trypsin (Sigma Chemical Co) to the zymogen at final concentrations of 91 μg/mL and 10 μg/mL, respectively. The resultant mixture was incubated for 30 minutes at 22 °C, and lima bean trypsin inhibitor (Sigma) was added at a final concentration of 30 μg/mL. The solvent was 0.15 mol/L of NaCl and 0.01 mol/L of $CaCl_2$ in 0.01 mol/L of tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5. The final product was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and exhibited three or four bands at mol wts of 80,000, 47,000, and 33,000 (product 1), or 80,000, 47,000, 33,000, and 23,000 (product 2), respectively.

Heparin. Porcine heparin was obtained either at an early stage in the manufacturing process prior to treatment of the mucopolysaccharide with oxidizing agents (Wilson Chemical Co, Chicago) or as pharmaceutical grade product with an anticoagulant activity of 169 USP U/mg (Diosynth, Inc, Chicago). Heparin purchased from the first supplier was further purified by cetylpyridinium chloride precipitation.[17,18] This mucopolysaccharide was then chromatographed on Sephadex G-100 to obtain low-mol-wt species subsequently affinity-fractionated with limiting amounts of antithrombin as described in our previous report.[4] The final product possessed an anticoagulant activity of 350 USP U/mg and exhibited an average mol wt of ~7,000 daltons (heparin-1). Mucopolysaccharide purchased from the second manufacturer was affinity-fractionated with saturating amounts of antithrombin-Sepharose 4B as outlined in a prior report.[21] The final product possessed a specific anticoagulant activity of 280 USP U/mg and exhibited an average mol wt of ~15,000 daltons (heparin-2).

Measurement of protein and mucopolysaccharide concentration. Protein concentrations of human factor IX and human antithrombin were determined by absorbance measurements at 280 nm using the extinction coefficients 13.2[17,18] and 6.5,[22] respectively. Protein concentrations of human factor XI were determined according to the method of Lowry et al.[23] Heparin concentrations were quantitated colorimetrically by assay of uronic acid according to the

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method of Bitter and Muir[24] or by measurement of hexosamine as described by Balazs et al.[25] The relationship between these two parameters and the dry weight of heparin fractions was determined experimentally.

Assay of factor XIa activity. The biologic activity of factor XIa was quantitated by its ability to release radiolabeled activation peptide from tritiated factor IX. The human factor IX used for this purpose was radiolabeled by oxidation of carbohydrate units present within the protein and subsequent reduction of these moieties with sodium ^{3}H-borohydride (7.3 Ci/mmol, New England Nuclear, Boston) according to the method of Silverberg et al.[26] The tritiated factor IX product exhibited a specific activity of 2.3×10^{8} cpm/mg. The assays were initiated by combining 25 μL of factor XIa at a concentration of up to 1.65×10^{-9} mol/L in a mixture of polybrene (Aldrich Chemical Co), bovine serum albumin (BSA) (Sigma), and $CaCl_2$ at levels of 0.2 mg/mL, 0.6 mg/mL, and 0.02 mol/L, respectively, with 25 μL of a mixture of tritiated factor IX and BSA at concentrations of 35 μL/mL and 1 mg/mL, respectively. The environmental conditions used for both solutions were 0.15 mol/L NaCl in 0.01 mol/L of Tris-HCl, pH 7.5. The reaction mixture was incubated for six to eight minutes at 37 °C. Zymogen conversion was quenched by the addition of 50 μL of 9.4% trichloracetic acid, and the resulting solution was centrifuged at 3,200 g at 4 °C for five minutes. The peptide liberated during the activation of factor IX was quantitated by measuring tritium counts in 80 μL of the supernatant. A calibration curve was constructed using various dilutions of a factor XIa solution of known amount ranging from 8.25×10^{-10} mol/L to 1.65×10^{-9} mol/L. A linear relationship was observed between the concentration of added enzyme and the level of activation peptide. The associated *r* value averaged .98 and the coefficient of variation for a given set of assays was ~3%.

Kinetic evlauation of the factor XIa–antithrombin interaction. The kinetics of the factor XIa–antithrombin interaction were examined by determining the residual level of enzymatic activity as a function of the time of incubation with protease inhibitor. The effective molar concentration of factor XIa was established by titrating the above enzyme with varying levels of antithrombin in the presence of saturating amounts of mucopolysaccharide. The loss of factor XIa activity was a linear function of antithrombin concentration, and its extrapolation to 100% inactivation was used to compute the effective molar level of enzyme with regard to protease inhibitor. The experimentally determined concentration of factor XIa was 85% (product 1) and 37% (product 2) of the value calculated from the known stock concentrations of enzyme, assuming a 2:1 molar stoichiometry of antithrombin to factor XIa (11). For all studies, the effective concentration of factor XIa was computed by multiplying the precentage of active species by the stock level of enzyme. Single reaction mixtures were prepared with the initial concentrations of factor XIa, antithrombin, and heparin of 5.76×10^{-8} mol/L, 5.76×10^{-6} mol/L, and 5.88×10^{-6} mol/L or 0, respectively. All components were either dissolved in or extensively diluted with 0.15 mol/L of NaCl in 0.01 mol/L of tris-HCl, pH 7.5. All solutions were incubated at 37 °C. At five separate times that ranged from 10 seconds to 10 minutes, aliquots of 3.2 μL were removed from the central reservoir and diluted 35-fold into 0.6 mg/mL of BSA, 0.20 mg/mL of polybrene, 0.02 mol/L of $CaCl_2$, 0.15 mol/L of NaCl in 0.01 mol/L of Tris-HCl, pH 7.5. This procedure reduced the reaction velocity to nil and brought the enzyme concentration into the range of the peptide cleavage assay. Thereafter, each of the times was assayed in duplicate as previously described. The adsorption of factor XIa to the walls of the test tubes was estimated by performing the above studies with antithrombin omitted from the initial incubation mixture. The rate of loss of factor XIa was ~3% of the rate constant in the presence of heparin and ~50% of the rate constant in the absence of mucopolysaccharide. In all instances, the final kinetic data were corrected to eliminate these absorption phenomena.

The results obtained above were analyzed by fitting the initial level of enzyme (E_o) as well as all subsequent levels of residual enzyme (E) observed at time (t) to the equation: $\ln(E) = \ln(E_o) - Kt$, where K represents the pseudo first-order rate constant of this reaction. The times used in this procedure included the first 30% to 50% of the reaction, and associated *r* values ranged from .91 to .99.

SDS-PAGE evaluation of the factor XIa–antithrombin interaction. The kinetics of the factor XIa–antithrombin interaction were also examined by determining the amount of hemostatic enzyme–protease inhibitor complex formed at a given time with SDS-PAGE. To this end, fixed concentrations of antithrombin were incubated with specific levels of heparin or buffer for several minutes at 37 °C. Given concentrations of factor XIa were added to both of the above reaction mixtures, and the solutions were incubated for 15 minutes at 37 °C. The two samples were then mixed with an equal volume of 0.1% bromphenol blue, 10% glycerol, and 3% SDS in 60 mol/L Tris-HCl, pH 6.8 prior to heating at 100 °C for five minutes. The various molecular species present within the reaction mixtures were then examined by SDS-PAGE according to the method of Laemmli,[27] using a gel solution containing 7.3% acrylamide and 0.2% methylenebisacrylamide. Proteins were transferred to nitrocellulose paper according to minor modifications of the technique of Burnette.[28] For this purpose, the gel and a sheet of nitrocellulose (0.45-μm pore size, Schliecher and Schuell, Keene, NH) presoaked in 20% methanol and 0.15 mol/L of glycine in 20 mol/L of Tris were layered between two sheets of Whatman filter paper (No. 3) also presoaked in the above buffer. The "sandwich" was positioned in an electrophoretic blotting apparatus (Biorad), and a current of 200 mA was applied for 19 hours at 4 °C. The nitrocellulose paper containing the transferred proteins was soaked in 5% nonfat dry milk, 0.01% antifoam A, 0.05% Tween-20, and 2 mmol/L of $CaCl_2$ in 50 mmol/L of Tris-HCl,pH 8 (Blotto) for two hours at 24 °C with gentle agitation. The blot was incubated in fresh Blotto containing 90.0 μg/mL (final concentration) of goat antisera directed against human antithrombin (Atlantic Antibodies, Portland, Me) for 90 minutes at 20 °C with gentle agitation. The nitrocellulose paper was then washed several times with Blotto and incubated with fresh Blotto containing 3 μg/mL (final concentration) of peroxidase-conjugated rabbit anti-goat IgG (Cappel, Cochranville, Pa) for one hour at 24 °C with constant agitation. The blot was rinsed several times with Blotto and with 0.15 mol/L NaCl in 0.01 mol/L of sodium phosphate, pH 7.4 (PBS). It was then soaked in 0.05% hydrogen peroxide in 5 vol of PBS and 1 vol of 4-chloro-1-naphthol in methanol and developed for 40 minutes at 24 °C. The reaction was quenched by washing the blot several times in distilled water. The blot was then dried and photographed.

RESULTS AND DISCUSSION

The inhibition of human factor XIa by human antithrombin was studied at 37 °C in the presence and absence of heparin. To examine the interaction in the absence of heparin, factor XIa (product 1, 85% active with respect to its interactions with antithrombin) was incubated with antithrombin for varying lengths of time at final concentrations of 5.76×10^{-8} mol/L and 5.76×10^{-6} mol/L respectively, and the amount of residual enzyme was estimated as a function of time through its ability to cleave tritiated human factor IX. The data have been corrected for losses due to instability of factor XIa and/or its adsorption to the walls of test tubes by conduction of kinetic experiments similar to those outlined above except that antithrombin was replaced by identical levels of BSA. A time-dependent decay in the biologic activity of factor XIa is observed with ∼75% of the enzyme remaining after ten minutes of incubation (Fig 1A).

We have also carried out additional kinetic experiments with a second source of factor XIa (product 2, 37% active with regard to its interactions with antithrombin) as well as with the same serine protease inhibitor product described above. No significant differences in the rate of factor XIa inactivation by antithrombin were noted with the two forms of the enzyme. The above results are similar to those reported earlier.[7,11] The pseudo first-order rate constant for six sets of experiments with both forms of factor XIa averaged 0.025 min^{-1} after correction for enzyme loss due to instability and/or vessel wall adsorption.

To analyze the interaction in the presence of heparin, factor XIa (product 1) was incubated with antithrombin and affinity-fractionated mucopolysaccharide of 7,000 mol wt (heparin 1) for varying lengths of time at final concentrations of 5.76×10^{-8} mol/L, 5.76×10^{-6} mol/L and 5.88×10^{-6} mol/L, respectively, and the amount of residual enzyme was estimated as a function of time through its ability to cleave tritiated factor IX. The data have been corrected for losses due to instability of factor XIa and/or its adsorption to the walls of test tubes by conduction of kinetic experiments similar to those outlined above, except that antithrombin was replaced by identical levels of BSA. A very rapid time-dependent decay in the biologic activity of factor XIa is observed with about 50% of the enzyme remaining after 45 seconds of incubation (Fig 1B). Thus, the factor XIa–antithrombin interaction appears to be accelerated ∼40 fold by the addition of heparin. Similar experiments have been conducted using affinity-fractionated heparin of 15,000 mol wt (heparin 2) with virtually identical results (data not shown).

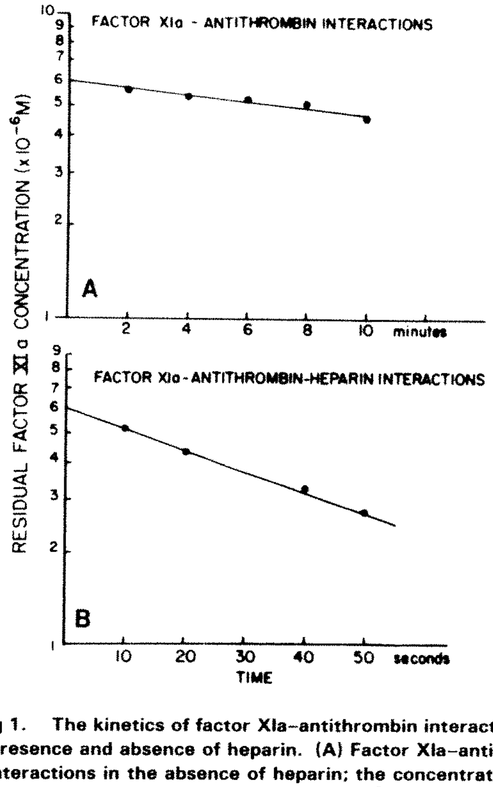

Fig 1. The kinetics of factor XIa–antithrombin interactions in the presence and absence of heparin. (A) Factor XIa–antithrombin interactions in the absence of heparin; the concentrations of factor XIa and antithrombin were 5.76×10^{-8} mol/L and 5.76×10^{-6} mol/L, respectively. (B) factor XIa–antithrombin interactions in the presence of heparin; the concentrations of factor XIa, antithrombin, and heparin were 5.76×10^{-8} mol/L, 5.76×10^{-6} mol/L, and 5.88×10^{-6} mol/L, respectively.

We have also carried out additional kinetic experiments with a second source of factor XIa (product 2) as well as with the same preparation of serine protease used above in the presence of the two types of affinity-fractionated mucopolysaccharide. No significant differences in the rates of factor XIa inactivation by antithrombin were noted with the two forms of the enzyme or heparin. Again, these results are in complete agreement with data reported in previous communications.[7,11] The pseudo first-order rate constant for 12 sets of experiments with both forms of factor XIa and mucopolysaccharide averaged 1.0 min^{-1} after correction for enzyme losses due to instability and/or vessel wall adsorption. Direct comparison of the rate constants of the factor XIa-antithrombin interactions in the presence and absence of heparin indicate that addition of either type of mucopolysaccharide leads to an ∼40-fold acceleration of enzyme neutralization by the protease inhibitor.

The acceleration of factor XIa–antithrombin complex formation using the well-defined preparation of low-mol-wt heparin (heparin 1) was examined using SDS-PAGE and Western blot analysis with antisera directed against the protease inhibitor. Purified human antithrombin and heparin were admixed at final concentrations of 1.13×10^{-6} mol/L and 1.43×10^{-6} mol/L, respectively, and incubated for several minutes at 37 °C. Purified human factor XIa was added to the above reaction mixture or to protease inhibitor in the absence of the mucopolysaccharide at a final concentration of 1.41×10^{-7} mol/L, and the solutions were incubated for 15 minutes at 37 °C. The concentrations of reactants and the incubation interval used were calculated from the kinetic data provided above so that the amount of hemostatic enzyme–protease inhibitor complex generated would be maximal in the presence of heparin and essentially nil in the absence of the mucopolysaccharide. As shown in Fig 2, the protease inhibitor migrated as a single band with a mol wt of 56,000 (lanes 1 and 4). A band with an apparent mol wt of >200,000 was observed in addition to that of antithrombin when factor XIa was incubated for 15 minutes with the protease inhibitor in the presence of heparin (lane 3). However, addition of the enzyme and antithrombin in the absence of the mucopolysaccharide for an identical period of time did not result in detectable complex formation between factor XIa and protease inhibitor (lane 2). These results are in excellent agreement with the studies using human factor XIa reported earlier by Kurachi and Davie.[11]

The interactions of human factor XIa with human antithrombin have been examined in the presence and absence of well-defined preparations of affinity-fractionated heparin from two commercial sources. The neutralization of factor

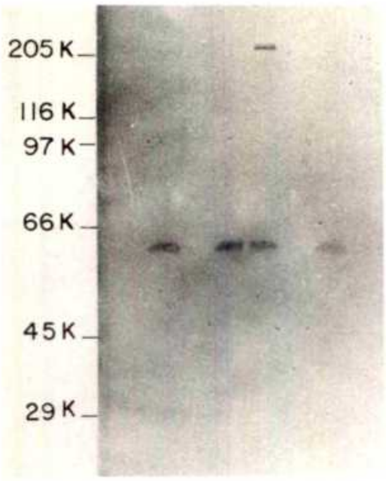

Fig 2. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of factor XIa–antithrombin interactions in the presence and absence of heparin. SDS-PAGE and Western blot analysis were performed as described in the text. Samples included antithrombin, lanes 1 and 4; factor XIa and antithrombin incubated for 15 minutes in the absence of heparin at concentrations of 1.41×10^{-7} mol/L, and 1.13×10^{-6} mol/L, respectively, lane 2; factor XIa and antithrombin incubated for 15 minutes in the presence of heparin, at concentrations of 1.41×10^{-7} mol/L, 1.13×10^{-6} mol/L, and 1.43×10^{-6} mol/L, respectively, lane 3. The mol wt standards, myosin (205,000 daltons), β-galactosidase (116,000 daltons), phosphorylase (97,000 daltons), bovine serum albumin (66,000 daltons), ovalbumin (45,000), and carbonic anhydrase (29,000 daltons), were electrophoresed in parallel with the above samples. The lanes containing the mol wt markers were separated from the gel prior to electrophoretic transfer of the coagulation proteins to nitrocellulose paper, stained with Coomassie brilliant blue (Sigma), and destained as outlined previously by Laemmli.[27] Shrinkage of the gel during electrotransfer of the proteins to the nitrocellulose paper was determined, and migration of proteins was corrected with respect to the migration of the mol wt standards.

XIa by antithrombin at concentrations of 5.76×10^{-8} mol/L and 5.76×10^{-6} mol/L, respectively, in the absence of mucopolysaccharide is slow with a pseudo first-order rate constant of 0.025 min^{-1}. The above estimate is in reasonable agreement with previously published values of this parameter.[29] The inhibition of factor XIa by antithrombin at similar concentrations of proteins, but in the presence of saturating levels of heparin, is accelerated dramatically with a pseudo first-order rate constant of 1.0 min^{-1}. Thus, the rate of enzyme–inhibitor complex formation appears to be enhanced by about ~40 fold in the presence of the mucopolysaccharide. These data were confirmed in a qualitative manner by direct monitoring of the generation of factor XIa–antithrombin interaction product wih SDS-PAGE and Western blot analysis using an antibody population specific for the protease inhibitor. The results obtained demonstrate formation of significant amounts of factor XIa–antithrombin complex in the presence of heparin, but essentially no interaction product in the absence of mucopolysaccharide.

Recently, Scott et al[19] reported that heparin concentrations of 1 U/mL did not enhance the rate of generation of the factor XIa–antithrombin interaction product, and that only a fourfold acceleration in the speed of hemostatic enzyme–protease inhibitor complex formation is observed in the presence of mucopolysaccharide levels of 5 to 30 U/mL. Based on earlier observations which indicated that α-1 antitrypsin is the major plasma protease inhibitor of factor XIa in the absence of mucopolysaccharide,[29,30] these investigators concluded that the heparin–antithrombin system is of relatively little physiologic importance in the neutralization of this hemostatic enzyme in the blood. However, the rate of factor XIa inhibition by the heparin–antithrombin complex observed by these authors and the data reported here differ by an order of magnitude. We suspect that the lack of stoichiometric titrations of factor XIa against antithrombin, the use of commercial sources of protease inhibitor, and the use of a relatively nonspecific esterolytic substrate may have contributed to the underestimation of the true rate of mucopolysaccharide-dependent acceleration of factor XIa–antithrombin interactions. Our estimates of the actual velocity of this reaction indicate that plasma concentrations of antithrombin completely saturated with heparin would become the dominant factor in the inhibition of factor XIa even at the relatively high levels of α-1 antitrypsin normally present in the blood. However, we did not quantitate the relative amounts of complex formed between the hemostatic enzyme and the various blood protease inhibitors by adding factor XIa to plasma containing heparin.

During the last few years, we have shown that macrovascular as well as microvascular bovine and rat endothelial cells grown in vitro are able to synthesize heparinlike molecules with potent anticoagulant activity and place these complex carbohydrates on their surfaces.[31-35] In addition, we have proven that these mucopolysaccharides contain the specific binding domains for antithrombin which are present in commercially available preparations of this complex carbohydrate.[34] Furthermore, we have demonstrated that these biologically active heparinlike molecules are present on the luminal surface of the vascular endothelium by perfusing rat and mouse hindlimb vasculature with purified thrombin as well as antithrombin and detecting a mucopolysaccharide-dependent acceleration of enzyme-inhibitor complex formation within the rodent circulatory system.[33,35] Given the anatomical location of anticoagulantly potent heparinlike molecules, and the dramatic enhancement in the rate of inhibition of the serine proteases of the intrinsic coagulation cascade by antithrombin bound to mucopolysaccharide, we have proposed that the presence of this specific complex carbohydrate on the surface of the endothelium constitutes an important mechanism for regulating the activity of the hemostatic system.[31-35] Although the rate of factor XIa inactivation by antithrombin in the presence of heparin is much less than kinetic constants reported for factors Xa and IXa as well as for thrombin, the kinetic data presented in this communication strongly indicate that the above model of the heparin–antithrombin mechanism is relevant to the in vivo neutralization of factor XIa generated within the circulatory tree. Similar kinetic data have not yet been determined for the other intrinsic clotting enzymes: factor XIIa and kallikrein. Finally, we are particularly intrigued by the 2:1

stoichiometry of this reaction, which leads to the dependence of the rate of inhibition of this enzyme on the square of the concentration of the heparin–antithrombin complex. These kinetic phenomena suggest that the positions and amounts of protease inhibitor bound to the heparinlike molecules of the endothelium may determine the rate of inhibition of factor XIa in the vascular system in a particularly dynamically balanced manner.

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REFERENCES

1. Rosenberg RD, Damus PS: The purification and mechanism of action of human antithrombin-heparin cofactor. J Biol Chem 248:6490, 1973
2. Murano G, Williams L, Miller-Andersson M, Aronson D, King C: Some properties of antithrombin III and its concentration in human plasma. Thromb Res 18:259, 1980
3. Rosenberg RD: Heparin action. Circulation 49:603, 1973
4. Jordan RE, Beeler D, Rosenberg RD: Fractionation of low molecular weight heparin species and their interaction with antithrombin. J Biol Chem 254:2902, 1979
5. Oosta GM, Gardner WT, Beeler DL, Rosenberg RD: Multiple functional domains of the heparin molecule. Proc Natl Acad Sci USA 78:829, 1981
6. Rosenberg JS, McKenna P, Rosenberg RD: Inhibition of human factor IXa by human antithrombin-heparin cofactor. J Biol Chem 250:8883, 1975
7. Damus PS, Hicks M, Rosenberg RD: A generalized view of heparin's anticoagulant action. Nature 246:355, 1973
8. Stead N, Kaplan AP, Rosenberg RD: Inhibition of activated factor XII by antithrombin-heparin cofactor. J Biol Chem 251:6481, 1976
9. Kurachi F, Fujikawa K, Schmer G, Davie EW: Inhibition of bovine factor IXa and factor Xa by antithrombin III. Biochemistry 15:373, 1976
10. Fujikawa K, Kurachi K, Davie EW: Characterization of bovine factor XIIa (activated Hageman factor). Biochemistry 16:4182, 1977
11. Kurachi K, Davie EW: Activation of human factor XI (plasma thromboplastin antecedent) by factor XIIa (activated Hageman factor). Biochemistry 16:5831, 1977
12. DiScipo RG, Kurachi K, Davie EW: Activation of human factor IX (Christmas factor). J Clin Invest 61:1528, 1978
13. Chan J, Burrowes C, Habal F, Movat H: Inhibition of activated factor XII (Hageman factor) by antithrombin III. The effect of other plasma proteinase inhibitors. Biochem Biophys Res Commun 74:150, 1977
14. Seegers WH, Cole ER, Harmison CR, Monkhouse FC: Neutralization of auto-prothrombin C activity with antithrombin. Can J Biochem 42:359, 1964
15. Briggs R, Denson KWE, Akman N, Borrett R, Hadden M: Antithrombin III, antifactor Xa and heparin. Br J Haematol 19:283, 1970
16. Yin ET, Wessler S, Stoll PJ: Identity of plasma-activated factor X inhibitor with antithrombin III and heparin co-factor. J Biol Chem 246:3712, 1971
17. Jordan RE, Oosta GM, Gardner WT, Rosenberg RD: The kinetics of hemostatic enzyme-antithrombin interactions in the presence of low molecular weight heparin. J Biol Chem 255:10081, 1980
18. Jordan RE, Oosta GM, Gardner WT, Rosenberg RD: The binding of low molecular weight heparin to hemostatic enzymes. J Biol Chem 255:10073, 1980
19. Scott CF, Schapira M, Colman RW: Effect of heparin on the inactivation of human factor XIa by antithrombin III. Blood 60:940, 1982
20. Mannhalter C, Schiffman S, Jacobs A: Trypsin activation of human factor XI. J Biol Chem 255:2667, 1980
21. Hoover RD, Rosenberg RD, Haering W, Karnovsky MJ: Inhibition of rat arterial smooth muscle cell proliferation by heparin. Circ Res 90:372, 1980
22. Nordenman B, Nystrom C, Bjork I: The size and shape of human and bovine antithrombin III. Eur J Biochem 78:195, 1977
23. Lowry OH, Rosebrough NJ, Farr AL, Randall RG: Protein measurements with the Folin phenol reagent. J Biol Chem 193:265, 1951
24. Bitter T, Muir HM: A modified uronic acid carbazole reaction. Anal Biochem 4:330, 1962
25. Balazs EA, Berntsen KO, Karossa J, Swann DA: An automated method for the determination of hexosamines. Anal Biochem 12:559, 1965
26. Silverberg SA, Nemerson Y, Zur M: Kinetics of the activation of bovine coagulation factor X by components of the extrinsic pathway. Kinetic behavior of two-chain factor VII in the presence and absence of tissue factor. J Biol Chem 252:8481, 1977
27. Laemmli UK: Cleavage of the structural proteins during assembly on the head of the bacteriophage T$_4$. Nature 227:680, 1970
28. Burnette WN: "Western blotting." Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112:195, 1981
29. Scott CF, Schapira M, James HL, Canen AB, Colman RW: Inactivation of factor XIa by plasma protease inhibitor. J Clin Invest 69:844, 1982
30. Heck LW, Kaplan AP: Substrates for Hageman factor: I. Isolation and characterization of human factor XI (PTA) and inhibition of the activated enzyme by alpha$_1$-antitrypsin. J Exp Med 140:1615, 1974
31. Marcum JA, Fritze L, Galli SJ, Karp G, Rosenberg RD: Microvascular heparin-like species with anticoagulant activity. Am J Physiol 245:H725, 1983
32. Marcum JA, Rosenberg RD: Anticoagulantly active heparin-like molecules from vascular tissue. Biochemistry 23:1730, 1984
33. Marcum JA, McKenney JB, Rosenberg RD: The acceleration of thrombin–antithrombin complex formation in rat hindquarters via heparin-like molecules bound to the endothelium. J Clin Invest 74:1, 1984
34. Marcum JA, Rosenberg RD: Heparinlike molecules with anticoagulant activity are synthesized by cultured endothelial cells. Biochem Biophys Res Commun 126:365, 1985
35. Marcum JA, McKenney JB, Galli SJ, Jackman RW, Rosenberg RD: Anticoagulantly active heparinlike molecules from mast cell-deficient mice. Am J Physiol (in press)

Influence of Fibrin and Liver Blood Flow on the Turnover and the Systemic Fibrinogenolytic Effects of Recombinant Human Tissue-Type Plasminogen Activator in Rabbits

By Henri Bounameaux, Jean-Marie Stassen, Conrad Seghers, and Désiré Collen

The influence of the presence of fibrin microclots on the systemic fibrinogenolytic effects of intravenous (IV) recombinant human tissue-type plasminogen activator (rt-PA) was studied by injection of a homogenized fibrin suspension in the femoral vein or artery in rabbits. A linear correlation ($P < .001$) was found between the extent of fibrinogen breakdown and the amount of fibrin (0 to 32 mg/kg) injected just prior to the IV infusion of rt-PA at a rate of 10 µg/kg/min for 60 minutes. This finding suggests that the systemic activation of the fibrinolytic system observed in some patients during infusion of rt-PA may be due, at least in part, to the presence of fibrin in the vascular bed. The effect of blood flow in the liver on the turnover of rt-PA was measured in rabbits after ligation of the hepatic artery and monitoring of the blood flow in the portal vein with a peristaltic pump. The half-life (t½) of rt-PA in plasma was inversely correlated with the logarithm of the rate of the liver blood flow. A doubling of the plasma t½ of rt-PA was observed after an eightfold reduction of the liver blood flow, suggesting that delayed clearance of rt-PA may occur in patients with severe cardiovascular failure and impaired liver blood flow.

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TISSUE-TYPE plasminogen activator (t-PA) is a key enzyme in physiological fibrinolysis.[1] It has a high affinity for plasminogen only in the presence of fibrin, thus allowing clot-selective fibrinolysis. Fibrin selectivity of t-PA has been demonstrated in vitro[2-4] as well as in several animal models of thrombosis[5-13] and in patients.[14,15] Recent multicenter clinical trials of t-PA obtained by recombinant DNA technology (rt-PA)[16] in patients with acute myocardial infarction[15,17-19] have confirmed its thrombolytic efficacy. However, in some patients an extensive systemic activation of the fibrinolytic system, only weakly correlated with the plasma steady-state levels of rt-PA, was observed.[20] It is possible that the presence of fibrin in the microcirculation contributes to systemic activation of the fibrinolytic system, resulting in fibrinogen breakdown.

In addition, a large interindividual variability in steady-state levels of rt-PA in plasma was observed in response to a constant infusion rate of rt-PA in patients.[17,19-21] Because t-PA is eliminated almost exclusively by the liver,[22,23] variability in liver blood flow may influence the clearance rate of rt-PA and result in variable blood levels.

In the present study, we investigated the influence of the presence of fibrin in the circulation on the systemic fibrinogenolytic effects of rt-PA and the influence of liver blood flow on its turnover rate in rabbits.

MATERIALS AND METHODS

Single chain rt-PA was obtained from Genentech Inc, South San Francisco, in a solution containing 1 mg (500,000 IU)/ml. In some experiments, it was labeled with ^{125}-Iodine.[24] Fibrin was prepared as follows: 1 mL of citrated rabbit plasma (final concentration of citrate 0.017 mol/L) containing trace amounts of ^{125}I-labeled fibrinogen were clotted by adding calcium chloride (final concentration 30 mmol/L) and bovine thrombin (4 NIH U/mL) (Hoffman-La Roche, Basel, Switzerland). After incubation for 30 minutes at 37 °C, the clot was blotted between two filter papers and washed several times with 0.15 mol/L of NaCl containing 1 IU/mL of heparin (Liquemin, Hoffman-La Roche). The fibrin gel was then homogenized and suspended with an ultrashear mixer (Polytron, Kinematica, Kriens LU, Switzerland), and its content of ^{125}I was measured.

New Zealand white rabbits with a body weight of 2.2 ± 0.1 kg (mean ± SEM) were anesthetized by intramuscular (IM) injection of 0.5 mL of Hypnorm (Duphar, Amsterdam, The Netherlands)—containing 10 mg/mL of fluanisone and 0.2 mg/mL of fentanyl—per kilogram of body weight. Additional Hypnorm (0.3 mL IM every two hours) was administered to maintain anesthesia. For the liver blood flow studies, which necessitated laparotomy, anesthesia was obtained with 0.8 mL of Hypnorm IM and pentobarbital at an intravenous (IV) dose of 5 mg/kg with maintenance injecions of 5 mg/kg every hour. Thyroidal uptake of radioiodide was blocked by administration of sodium iodide (0.5 mL IV of a 2% solution). A femoral vein catheter was introduced for blood sampling.

A suspension of radiolabeled fibrin (containing $\sim$500,000 cpm) was injected for approximately ten minutes in the femoral vein or artery on the contralateral side of the blood-sampling catheter. At the end of the fibrin infusion, rt-PA was infused over 60 minutes via a catheter placed in a marginal ear vein at a rate of 10 µg/kg/min. One-milliliter blood samples were drawn at fixed time intervals into trisodium citrate (final concentration 0.017 mol/L) and into trisodium citrate containing 200 KIU of aprotinin (Trasylol, Bayer, Leverkusen, F.R.G.) for measurement of plasma fibrinogen, α_2-antiplasmin, and rt-PA antigen. Whole blood radioactivity was counted before centrifugation. The extent of fibrinolysis at time t (FL), expressed in percentage, was calculated using the following formula: $FL = 100 \cdot A \cdot W \cdot 66 \cdot 3/B$, where A represents the radioactivity measured in 1 mL of blood at time t, W is the body wieght of the animal, and B is the total radioactivity injected. A blood volume of 66 mL/kg was assumed on the basis of a measured plasma volume of 35 mL. A correction factor of 3 for the distribution volume of labeled fibrin degradation products was used to account for the diffusion to the extravascular space.

To evaluate the effect of blood flow through the liver on the t½ of rt-PA, laparotomy was performed with a 10-cm paramedian incision, and the hepatic hilus was exposed. After injection of 500 IU of heparin, the hepatic artery was ligated, and the portal vein cannulated in its proximal segment with a polyethylene cannula with an internal diameter of 1.5 mm. The blood was passed through a peristaltic pump (Minipulse II, Gilson Medical Electronics, France)

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and reinjected distally in the portal vein at a variable rate. The volume of this extracorporeal loop was ~5 mL. The normal portal flow after ligation of the hepatic artery (hereafter referred to as liver blood flow) was 430 ± 60 mL/kg/h (mean ± SEM) as determined in six rabbits. Immediately after the surgical procedure, radiolabeled rt-PA was injected in a marginal ear vein as a bolus of 0.2 mg/kg (containing ~1,500,000 cpm). Blood samples were drawn at fixed time intervals into trisodium citrate for measurement of whole blood radioactivity and of rt-PA antigen in plasma.

The distribution volume of rt-PA was measured by injection of 0.2 mg/kg of rt-PA containing 1,500,000 cpm of labeled rt-PA in 20 seconds in five rabbits. Blood samples were taken after 30 and 60 seconds for measurement of whole blood radioactivity and plasma rt-PA antigen level; distribution volume of ^{125}I and rt-PA antigen was calculated from the dilution factor.

rt-PA antigen was determined using an enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies[25] and expressed in micrograms per milliliter by comparison with the International Reference Preparation for t-PA (83/517) obtained from Dr P.J. Gaffney, National Institute for Biological Standards and Control, London. This International Reference Preparation contains 1,000 IU per vial corresponding to 2 μg of t-PA. Fibrinogen was assayed in the plasma samples containing aprotinin using the method of Clauss[26] modified as previously described[27]; α_2-antiplasmin activity was assayed with the chromogenic substrate S-2251.[28] Unless stated otherwise, all values are given as means ± SEM. Statistical analysis was performed by using Student's *t* test for unpaired values or linear regression analysis when appropriate.[29]

RESULTS

The infusion of rt-PA at a rate of 10 μg/kg/min over 60 minutes in rabbits resulted in an average plasma steady-state concentration of 0.8 ± 0.2 μg/mL, which is similar to that obtained in humans.[20,21] IV or intraarterial (IA) injection of a homogenized fibrin suspension did not induce a significant change in the plasma levels of fibrinogen or α_2-antiplasmin (Fig 1). Infusion of rt-PA (10 μg/kg/min during 60 minutes) in control rabbits without fibrin injection caused a decrease of plasma fibrinogen by only 7% and of α_2-antiplasmin by 22%. Infusion of the same amount of rt-PA in rabbits subjected to prior injections of increasing amounts of the fibrin suspension (0 to 32 mg/kg), resulted in a dose-dependent increase of fibrinogen breakdown and α_2-antiplasmin consumption (Fig 1). The extent of fibrinogen breakdown at the end of the 60-minute infusion of rt-PA was linearly correlated with the injected amount of fibrin both after its IV (r = .86, n = 20, $P < .001$) or IA (r = .77, n = 13, $P < .01$) injection (Fig 2). The correlation between the amount of injected fibrin and the extent of α_2-antiplasmin consumption was not significant ($P > .05$), due to a large interindividual variability in response. Infusion of rt-PA resulted in a significant degree of fibrinolysis, as demonstrated by the increase of whole blood radioactivity in the treated rabbits as compared with the control animals (Fig 3).

The blood and plasma volumes determined from the dilution factor after injection of 0.2 mg/kg of radiolabeled rt-PA in five rabbits were 79 ± 1.5 mL/kg and 54 ± 4 mL/kg, respectively.

The decrease of liver blood flow to 50%, 25% and 12.5% of the normal value resulted in a prolongation of the t½ of rt-PA from 3.4 minutes (100%) to 3.9, 4.7, and 6.1 minutes, respectively, as determined from rt-PA antigen measurements in plasma (Fig 4A). The t½ of rt-PA determined from the disappearance rate of ^{125}I from blood were 3.6 minutes (100% liver blood flow), 5.2 (50%), 6.4 (25%), and 7.7 minutes (12.5%) (Fig 4B). The leveling off of the plasma disappearance curves of the radiolabel has been shown to be due to the reappearance in blood of radioactive degradation products of t-PA.[22] A negative correlation was found between the plasma t½ of rt-PA and the logarithm of the liver blood flow (Fig 5) (r = .67 for t-PA antigen and 0.78 for ^{125}I, n = 24, $P < .001$).

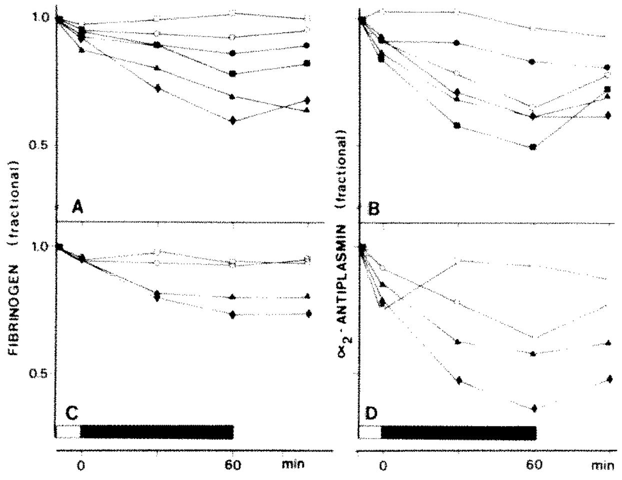

Fig 1. Effect of (A, B) intravenous or (C, D) intraarterial injection of a fibrin suspension on fibrinogen breakdown and α_2-antiplasmin consumption during infusion of rt-PA: ○, no fibrin; ●, 4 mg/kg; ■, 8 mg/kg; ▲, 16 mg/kg; ◆, fibrin 32 mg/kg of body weight; □, controls without rt-PA after injection of 32 mg/kg of fibrin. Horizontal open and closed bars represent injection of the fibrin suspension and infusion of rt-PA, respectively. The data are means of three or four experiments.

DISCUSSION

t-PA is presently under extensive investigation as a fibrin-specific thrombolytic agent in patients with acute myocardial infarction. It has been shown to be an efficient thrombolytic agent in humans,[15,17-19] but with the currently used infusion rates, variable degrees of systemic activation of the fibrinolytic system are observed.[20,21] The extent of fibrinogenolysis correlates only weakly with the plasma rt-PA level,[20] indicating that its mechanism is more complex than would be anticipated from the kinetics of the activation of plasminogen by t-PA.[30]

The results of the present study demonstrate that the presence of fibrin in the microcirculation promotes systemic fibrinolytic activation induced by rt-PA. The extent of fibrinogen breakdown was found to be directly proportional to the amount of injected fibrin. A highly significant linear correlation was observed in the range of 0 to 32 mg/kg; saturation at higher amounts cannot be excluded, however. This finding suggests that a low-grade disseminated intravascular coagulation, even if compensated, may contribute to systemic activation of the fibrinolytic system. A more exten-

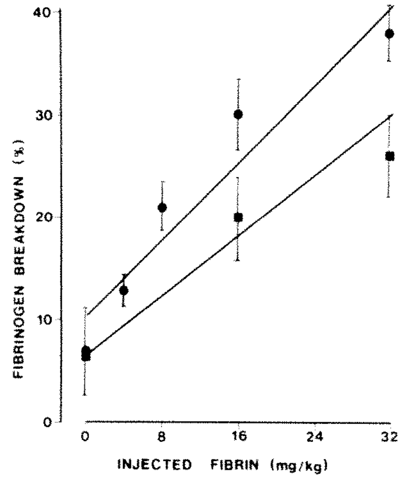

Fig 2. Correlation between extent of fibrinogen breakdwon (in percentage of baseline) and amount of injected fibrin: ●, intravenous injection of fibrin; ■, intraarterial injection of fibrin.

sive fibrinogen breakdown has recently been observed[31] during infusions of rt-PA in patients with deep vein thrombosis (in whom the amount of fibrin in the vascular bed would be large) than in patients with coronary thrombosis. This observation indeed supports the hypothesis that the amount of fibrin in the bloodstream correlates with the extent of fibrinogen degradation during rt-PA infusion in humans.

An unexpected observation, however, is that considerable fibrinogen breakdown (decrease to ~60% of the preinfusion level) can occur in the presence of residual levels of 60% of α_2-antiplasmin. We previously reported that systemic fibrinogen breakdown during infusion of streptokinase, urokinase, or plasmin in patients with thromboembolic disease occurs only in association with very low levels of residual α_2-antiplasmin.[32]

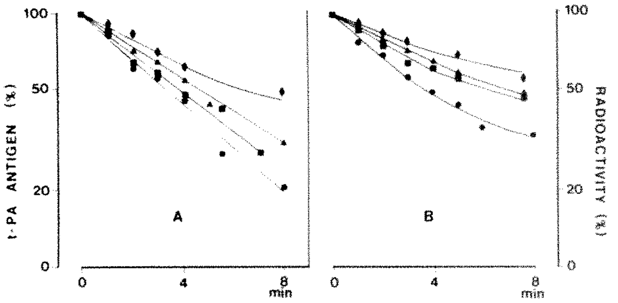

Fig 4. Effect of liver blood flow on the plasma disappearance rate of rt-PA following a bolus injection of a mixture of 0.2 mg/kg of rt-PA and 1,500,000 cpm of ^{125}I-labeled rt-PA: ●, 100% liver blood flow; ■, 50%; ▲, 25%; ◆, 12.5%. (A) rt-PA antigen in plasma; (B) ^{125}I in whole blood. Data expressed in percentage of the first sample are means of six experiments.

Another problem that emerged from the clinical trials of rt-PA in acute myocardial infarction was the large interindividual variability of the steady-state concentrations of rt-PA antigen following its infusion at a constant rate. The variability in plasma rt-PA level may be due to differences in the distribution volume of rt-PA or in its turnover. Our results in rabbits showed only minimal differences in the distribution volume of rt-PA as measured by bolus injection. Whether this observation can be extrapolated to patients with thromboembolic disease is questionable, however.

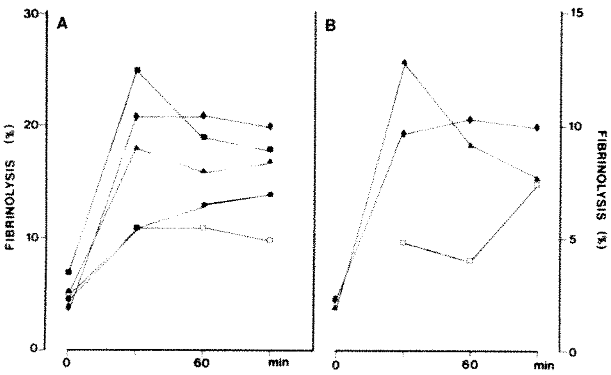

Fig 3. Extent of fibrinolysis (expressed in percentage) during infusion of rt-PA (10 μg/kg/min for 60 minutes) following intravenous (A) or intraarterial (B) injection of different amounts of a fibrin suspension: ●, 4 mg/kg; ■, 8 mg/kg; ▲, 16 mg/kg; ◆, 32 mg/kg; □, controls without rt-PA but after injection of 32 mg/kg of fibrin. The data are means of three or four experiments.

Diminished hepatic blood flow results in delayed hepatic clearance of a wide variety of agents from the circulation.[33] Because rt-PA is eliminated almost exclusively by the liver,[22,23] we studied the turnover of radiolabeled rt-PA in rabbits with mechanically impaired liver blood flow. The normal flow measured by direct cannulation of the portal vein after ligation of the hepatic artery was 430 ± 60 mL/kg/h. This value is comparable to the total hepatic flow in humans, which is about 800 mL/kg/h, as measured by

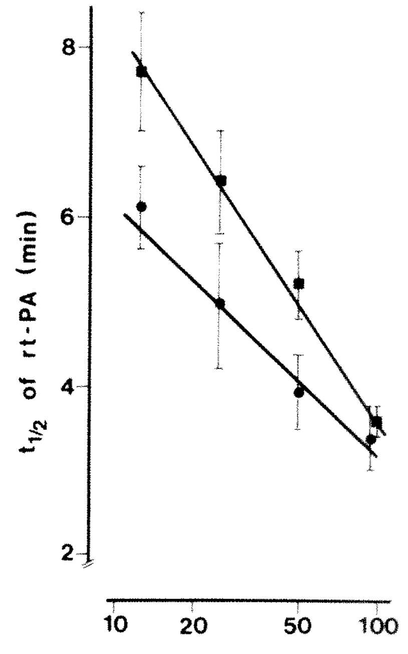

Fig 5. Correlation between the half-life (t½) of rt-PA (in minutes) and liver blood flow (in percentage of normal): ●, t½ of rt-PA antigen (as in Fig 4A); ■, t½ of the radiolabel (as in Fig 4B).

indocyanine green clearance.[34] The plasma t½ of rt-PA antigen was 3.4 ± 0.4 minutes during shunting of the blood flow through a peristaltic pump at the normal rate. This t½ is similar to that previously reported in intact rabbits.[22,35] An inverse relationship between the plasma t½ of rt-PA and the logarithm of liver blood flow was observed, with a reduction of liver blood flow to 12.5% of the normal flow resulting in a doubling of the t½. Thus, a very marked reduction in liver blood flow was required to obtain a significant prolongation of the plasma t½ of rt-PA. Such a reduction may, however, occur in patients with severe hemodynamic failure due to massive pulmonary embolism or shock, but is unlikely to occur in patients with uncomplicated myocardial infarction.

In summary, the present study identifies two factors that may contribute to the large interindividual variability in plasma rt-PA levels and extent of fibrinogen breakdown during rt-PA infusion. When fibrin is present in the vascular tree in concentrations between 5 and 30 mg/kg, systemic activation of the fibrinolytic system and fibrinogen breakdown is promoted. In addition, severe impairment of liver blood flow results in delayed clearance of rt-PA from the blood.

REFERENCES

1. Collen D: On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost 43:77, 1980

2. Korninger C, Collen D: Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro. Thromb Haemost 46:651, 1981

3. Lijnen HR, Marafino BJ Jr, Collen D: In vitro fibrinolytic activity of recombinant tissue-type plasminogen activator in the plasma of various primate species. Thromb Haemost 52:308, 1984

4. Mattson C, Nyberg-Arrhenius V, Wallén P: Dissolution of thrombi by tissue plasminogen activator, urokinase and streptokinase in an artificial circulating system. Thromb Res 21:535, 1981

5. Matsuo O, Rijken DC, Collen D: Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature 291:590, 1981

6. Korninger C, Matsuo O, Suy R, Stassen JM, Collen D: Thrombolysis with human extrinsic (tissue-type) plasminogen activator in dogs with femoral vein thrombosis. J Clin Invest 69:573, 1982

7. Collen D, Stassen JM, Verstraete M: Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. Effect of molecular form and dose of activator, age of the thrombus, and route of administration. J Clin Invest 71:368, 1983

8. Bergmann SR, Fox KAA, Ter-Pogossian MM, Sobel BE, Collen D: Clot-selective coronary thrombolysis with tissue-type plasminogen activator. Science 220:1181, 1983

9. Van de Werf F, Bergmann SR, Fox KAA, De Geest H, Hoyng CF, Sobel BE, Collen D: Coronary thrombolysis with intravenously administered human tissue-type plasminogen activator produced by recombinant DNA technology. Circulation 69:605, 1984

10. Flameng W, Van de Werf F, Vanhaecke J, Verstraete M, Collen D: Coronary thrombolysis and infarct-size reduction following intravenous infusion of recombinant tissue-type plasminogen activator in non-human primates. J Clin Invest 75:84, 1985

11. Carlin G, Einarsson M, Saldeen T: Tissue plasminogen activator effectively lyses intravascular fibrin deposits in the rat lung, in Davidson JF, Bachmann F, Bouvier CA, Kruithof EKO (eds): Progress in Fibrinolysis, vol 6. Edinburgh, Churchill Livingstone, 1983, p 471

12. Gold HK, Fallon JT, Yasuda T, Khaw BA, Newell JB, Leinbach RC, Guerrero JL, Vislosky FM, Hoyng CF, Grossbard E, Collen D: Coronary thrombolysis with recombinant human tissue plasminogen activator. Circulation 70:700, 1984

13. Topol EJ, Ciuffo AA, Dillman J, Builder S, Grossbard E, Weisfeldt ML, Bulkley BH: Thrombolysis with recombinant tissue plasminogen activator in atherosclerotic thrombotic occlusions. J Am Coll Cardiol 5:85, 1985

14. Van de Werf F, Ludbrook PA, Bergmann SR, Tiefenbrunn AJ, Fox KAA, De Geest H, Verstraete M, Collen D, Sobel BE: Coronary thrombolysis with tissue-type plasminogen activator in patients with evolving myocardial infarction. N Engl J Med 310:609, 1984

15. Collen D, Topol EJ, Tiefenbrunn AJ, Gold HK, Weisfeldt ML, Sobel BE, Leinbach RC, Brinker JA, Ludbrook PA, Yasuda T, Bulkley BH, Robison AK, Hutter AM Jr, Bell WR, Spadaro JJ Jr, Khaw BA, Grossbard E: Coronary thrombolysis with recombinant human tissue-type plasminogen activator: A prospective, randomized, placebo-controlled trial. Circulation 70:1012, 1984

16. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D: Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli*. Nature 301:224, 1983

17. Verstraete M, Bernard R, Bory M, Brower RW, Collen D, de Bono DP, Erbel R, Huhmann W, Lennane RJ, Lubsen J, Mathey D, Meyer J, Michels HR, Rutsch W, Schartl M, Schmidt W, Uebis R, von Essen R: Randomised trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. Lancet 1:842, 1985

18. TIMI Study Group: The thrombolysis in myocardial infarction (TIMI) trial. Phase I findings. N Engl J Med 312:932, 1985

19. Verstraete M, Bleifeld W, Brower RW, Charbonnier B, Collen D, de Bono DP, Dunning AJ, Lennane RJ, Lubsen J, Mathey DG, Michel PL, Raynaud Ph, Schofer J, Vahanian A, Vanhaecke J, Van de Kley GA, Van de Werf F, von Essen R: Double-blind randomised trial of intravenous tissue-type plasminogen activator versus placebo in acute myocardial infarction. Lancet 2:965, 1985

20. Collen D, Bounameaux H, De Cock F, Lijnen HR, Verstraete M: Analysis of coagulation and fibrinolysis during intravenous infusion of recombinant human tissue-type plasminogen activator (rt-PA) in patients with acute myocardial infarction. Circulation (in press)

21. Verstraete M, Bounameaux H, De Cock F, Van de Werf F, Collen D: Pharmacokinetics and systemic fibrinogenolytic effects of recombinant human tissue-type plasminogen activator (rt-PA) in man. J Pharmacol Exp Ther 235:506, 1985

22. Korninger C, Stassen JM, Collen D: Turnover of human extrinsic (tissue-type) plasminogen activator in rabbits. Thromb Haemost 46:658, 1981

23. Fuchs HE, Berger H, Pizzo SV: Catabolism of human tissue-plasminogen activator in mice. Blood 65:539, 1985

24. McFarlane AG: Efficient trace-labelling of proteins with iodine. Nature 182:53, 1958

25. Holvoet P, Cleemput H, Collen D: Assay of human tissue-type plasminogen activator (t-PA) with an enzyme-linked immunosorbent assay (ELISA) based on three murine monoclonal antibodies to t-PA. Thromb Haemost 54:684, 1958

26. Clauss A: Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol (Basel) 17:237, 1957

27. Vermylen C, De Vreker R, Verstraete M: A rapid method for

assay of fibrinogen fibrin polymerization time. Clin Chim Acta 8:418, 1963

28. Edy J, De Cock F, Collen D: Inhibition of plasmin by normal and antiplasmin-depleted plasma. Thromb Res 8:513, 1976

29. Snedecor GW, Cochran WG: Statistical Methods, 6th ed. Ames, Iowa, Iowa State University Press, 1973

30. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue-type plasminogen activator. Role of fibrin. J Biol Chem 257:2912, 1982

31. Turpie AGG, Jay RM, Carter CJ, Hirsh J: A randomized trial of recombinant tissue plasminogen activator for the treatment of proximal deep vein thrombosis. Circulation 72:III-193, 1985

32. Collen D, Verstraete M: α_2-Antiplasmin consumption and fibrinogen breakdown during thrombolytic therapy. Thromb Res 14:631, 1979

33. Lautt WW: Relationship between hepatic blood flow and overall metabolism: The hepatic arterial buffer response. Fed Proc 42:1662, 1983

34. Leevy CM, Mendenhall CL, Lesko W, Howard M: Estimation of hepatic blood flow with indocyanine green. J Clin Invest 41:1169, 1962

35. Bounameaux H, Verstraete M, Collen D: Pharmacokinetics of human tissue-type plasminogen activator (t-PA), in Verstraete M, Collen D, Lijnen HR, Verstraete M (eds): Thrombolysis: Biological and Therapeutic Properties of New Thrombolytic Agents. Edinburgh, Churchill Livingstone, 1985, p 85

Identification of Functional Domains on von Willebrand Factor by Binding of Tryptic Fragments to Collagen and to Platelets in the Presence of Ristocetin

By Wim P.M. Houdijk, Marion E. Schiphorst, and Jan J. Sixma

With the use of monoclonal antibodies that inhibit the ristocetin-induced binding of von Willebrand factor (VWF) to platelets and the binding to collagen, we have previously identified two distinct tryptic fragments. To prove that these fragments contain the platelet binding or the collagen binding domain, we investigated the direct binding of tryptic fragments of ^{125}I-VWF to platelets in the presence of ristocetin and to collagen fibrils. During the course of the tryptic digestion, there was a rapid and parallel decrease in binding to platelets and collagen. In the first ten minutes, binding decreased >50%; a further decrease to 19% and 29%, respectively, was noted at 90 minutes, but no further decrease was observed thereafter.

The bound fragments were eluted from platelets and collagen and analyzed on polyacrylamide gradient gels. The fragments bound to the platelets appeared to be reduced, probably by endogenous reducing substances from the platelets. This was prevented by addition of *N*-ethylmaleimide during the incubation. After 24 hours of digestion, platelets predominantly bound fragments of 116 kd and collagen bound a single fragment of 48 kd. These fragments are similar to those previously identified with the monoclonal antibodies.

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FVIII-VWF is a complex of two molecules: factor VIII (FVIII), the procoagulant molecule absent in hemophilia A, and von Willebrand factor (VWF) absent in von Willebrand's disease.[1] VWF is present as a series of multimers in which the repeating protomer is a dimer of the 250-kd primary subunit.[2-4] VWF is required for the adhesion of blood platelets to collagen and to subendothelium at high shear rates.[5,6] This adhesion-enhancing function of VWF is affected by binding of VWF to subendothelium or to collagen followed by interaction of blood platelets with this bound VWF.[6,7] The interaction of blood platelets with bound VWF occurs through the platelet membrane glycoprotein Ib, absent in the Bernard-Soulier syndrome.[8,9] The interaction of VWF bound to collagen or to subendothelium with blood platelets is in this respect similar to the binding of VWF to platelets in the presence of ristocetin. This ristocetin-induced binding is thus used as a model for the VWF activity, although both activities are not always parallel.[10]

The study of the structure–function relationship of a large multimeric molecule such as VWF is hampered by the fact that only low residual activity remains after disruption of the molecule.[11] In a previous report, we used two monoclonal antibodies against VWF to identify the platelet and collagen binding domains on VWF.[12] The platelet binding domain was associated with a 116-kd tryptic fragment, and the collagen binding domain was associated with a 48-kd tryptic fragment. Here we describe the direct binding of tryptic fragments of VWF to collagen and to platelets in the presence of ristocetin. These data demonstrate that the peptides previously identified by the monoclonal antibodies bind to collagen and to platelets. It becomes evident that VWF is a multidomain molecule with distinct separated binding domains for collagen and blood platelets.

MATERIALS AND METHODS

All chemicals were purchased from commercial sources and were of the highest purity grade available. Benzamidine, *N*-ethylmaleimide (NEM), and soybean trypsin inhibitor (type I-S) were from Sigma Chemical Co (St Louis). ε-aminocaproic acid was from Nogepha (Alkmaar, the Netherlands). L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin was obtained from Worthington Biochemical Corp (Freehold, NJ). Trichloroacetic acid (TCA) and sodium deoxycholate were from Merck AG (Darmstadt, F.R.G.). Carrier-free Na ^{125}I (13–17 Ci/mg) was purchased from Amersham International Ltd (Amersham, England). Iodo-Gen was from Pierce Chemical Co (Rockford, Ill). Ristocetin sulfate was obtained from H. Lundbeck and Co (Copenhagen). Acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED), β-mercaptoethanol, and ammoniumpersulfate were electrophoresis-grade reagents obtained from Bio-Rad Laboratories (Richmond, Calif).

Purification and radiolabeling of FVIII-VWF. FVIII-VWF was purified from human cryoprecipitates by gel filtration on Sepharose CL-4B.[13] The FVIII-VWF in the void volume fractions was precipitated by dialysis against 1.6 mol/L of ammonium sulfate, pH 7.0, at 4 °C. The precipitated protein was collected by centrifugation (two minutes, 10,000 g, 4 °C). The pellet was dissolved in 0.05 mol/L of Tris-HCl, and 0.1 mol/L of NaCl, pH 7.4, and further dialyzed against the same buffer. Radiolabeling with ^{125}I was performed with the Iodo-Gen method.[14] Free ^{125}I was removed by dialysis at 4 °C against 0.05 mol/L Tris and 0.1 mol/L of NaCl, pH 7.4. The labeled preparations had a specific activity of ~26 μCi/mg, and free ^{125}I was < 5%.

Tryptic digestion of FVIII-VWF. FVIII-VWF was digested with trypsin as previously described.[12] TPCK-trypsin was added to a solution of ^{125}I FVIII-VWF in 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4. The incubation was performed at 37 °C at an enzyme-substrate ratio of 1:2.2 (mol/mol). Before the addition of trypsin at ten minutes, 90 minutes, 6 hours, and 24 hours after the onset of digestion, samples were collected from the incubation mixture and added to a solution of inhibitors to give a final concentration of 12.5 mmol/L of benzamidine, 12.5 mmol/L of ε-aminocaproid acid, and a twofold molar excess of soybean trypsin inhibitor.

Binding of tryptic fragments to platelets. Human blood was collected into 1:10 vol of 110 mmol/L of trisodium citrate, and platelet-rich plasma was obtained by centrifugation (ten minutes,

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150 g, 20 °C). Platelets were washed three times by centrifugation as previously described.[7] After being washed, the platelets were resuspended in Tyrode's buffer (140 mmol/L of NaCl, 2.7 mmol/L of KCl, 0.42 mmol/L of NaH_2PO_4, 12 mmol/L of $NaHCO_3$), pH 7.4, containing 1 mmol/L of $CaCl_2$, 5 mmol/L of glucose, and 3.5 mg/mL of human albumin. ^{125}I-VWF or tryptic fragments of ^{125}I-VWF were diluted to a concentration of 20 μg/mL in Tyrode's solution containing $CaCl_2$ and albumin. A volume of 0.25 mL of this solution was first mixed with 4 μL of a ristocetin solution (200 mg/mL); 0.25 mL of the platelet suspension, containing 1.5×10^8 platelets, was then added. As a control, the incubation of ^{125}I-VWF and platelets was performed without ristocetin. After incubation for 30 minutes at room temperature, the mixture was layered on top of 1 mL of 20% (wt/vol) sucrose in Tyrode's buffer and then centrifuged (one minute, 10,000 g). The supernatant was aspirated, and the platelet pellet was counted in a γ counter.

The bound ^{125}I-VWF was eluted by resuspending the platelet pellet in Tyrode's containing 10 mmol/L of EDTA, immediately after centrifugation. The suspension was centrifuged and the supernatant contained >60% of the bound radioactivity. The eluted material was concentrated by TCA precipitation: 0.1 mL of 0.15% (wt/vol) sodium deoxycholate was added to 1 mL of the supernatant; after ten minutes, 0.1 mL 72% (wt/vol) TCA was added, and the precipitate was collected by centrifugation. More than 90% of the radioactivity was precipitated by TCA. The TCA pellet was boiled for five minutes in sample buffer (0.0625 mol/L of Tris-HCl, pH 6.8, 3% (wt/vol) sodium dodecyl sulfate (SDS) 10% (vol/vol) glycerol, 0.05% (wt/vol) bromophenol blue) and applied to a SDS polyacrylamide gradient gel. The platelet pellet was not directly solubilized in sample buffer to avoid overloading the gel with protein. Analysis of bound fragments by immunoprecipitation was performed as discussed previously.[12]

Binding of tryptic fragments to collagen. In this study, we used fibrils of collagen type III. The isolation of collagen type III from human umbilical arteries and the preparation of fibrils have been described previously.[6] ^{125}I-VWF or tryptic fragments of ^{125}I-VWF were diluted to a concentration of 20 μg/mL in 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4, containing 1% (wt/vol) bovine serum albumin (BSA). A volume of 0.25 mL of this solution was mixed with 0.2 mL of a suspension of collagen fibrils (1 mg/mL in 0.02 mol/L of Na_2HPO_4). As a control, 0.2 mL of sodium phosphate buffer was added. The mixture was incubated for 30 minutes at 37 °C and centrifuged (15 minutes, 10,000 g). The collagen pellet was washed once with 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, pH 7.4, and then resuspended in 0.1 mL of the same buffer containing 1% (wt/vol) SDS which eluted >85% of bound radioactivity. After centrifugation, the supernatant was electrophoresed on a SDS polyacrylamide gradient gel. Analysis of bound fragments by immunoprecipitation was performed as described previously.[12]

Polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed on 3% to 30% polyacrylamide gradient gels essentially according to Laemmli.[15] The gels were run at 200 V (constant voltage) for 20 hours, stained with Coomassie brilliant blue R-250, and dried. Autoradiography was performed with Kodak-X-Omat AR5 film (Eastman Kodak Co, Rochester, NY) and DuPont Cronex Lightning-plus intensifying screens (DuPont Instruments, Wilmington, Del). A mixture of low mol wt protein markers (Pharmacia, Uppsala, Sweden) and high mol wt protein markers (Bio-Rad) was run in parallel.

RESULTS

Binding of ^{125}I-VWF to platelets and collagen during tryptic degradation. Figure 1 shows the binding of ^{125}I-VWF to washed platelets in the presence of ristocetin or to collagen during the course of the digestion of ^{125}I-VWF with trypsin. Before digestion, and 10 minutes, 90 minutes, 6 hours, and 24 hours after the onset of digestion, samples were taken from the incubation mixture, inhibitors were added, and the binding of ^{125}I-VWF to platelets and collagen was determined. During the first 90 minutes, binding to both platelets and collagen rapidly declined with no further decrease thereafter. After ten minutes, the binding to platelets in the presence of ristocetin was decreased to 47% of the initial value, and the binding to collagen was decreased to 48%. From ten minutes to 90 minutes, the ristocetin-induced binding further decreased to 19% of the starting value, and the binding to collagen declined to 29%. After 24 hours, the binding to platelets was 15% of the binding before digestion and only two times above the control (platelets without ristocetin). The binding to collagen was 26% of the starting value and four times above the control.

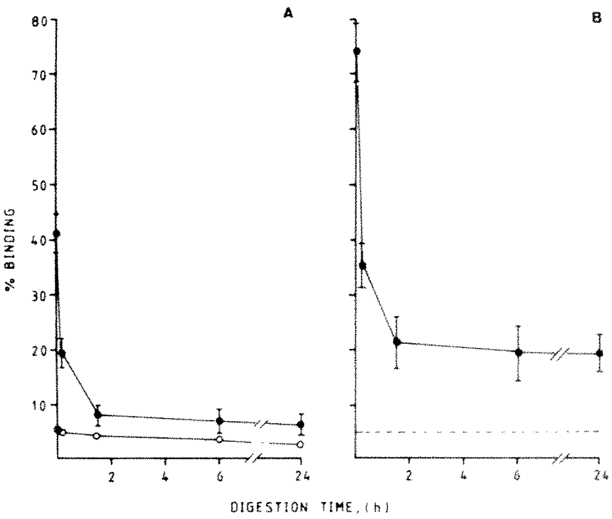

Fig 1. **Binding of ^{125}I-von Willebrand factor (VWF) to platelets and collagen during the course of digestion with trypsin. (A) Binding to platelets in the presence of ristocetin (●—●). At different times, samples from the digestion mixture were incubated with washed platelets and ristocetin. As a control, the incubation with platelets was performed without ristocetin (○—○). Values represent mean ± SEM; n = 3. (B) Binding to collagen. At the indicated times, samples were incubated with fibrils of collagen type III. Before addition of trypsin, a sample of ^{125}I-VWF was also incubated without collagen. This binding is indicated by the dashed line. Values are mean ± SEM; n = 6.**

The rapid and parallel decline of the binding to platelets and collagen was accompanied by a degradation of the VWF molecule. The tryptic degradation pattern of VWF is shown in Fig 2 and has been described previously.[12] After ten minutes, a 36- to 34-kd doublet appeared, and high mol wt material entered the gel. At 90 minutes, fragments of 180, 116, and 48 kd appeared. At 24 hours, two fragments of 116 and 48 kd were predominantly present.

Tryptic fragments of ^{125}I-VWF binding to platelets in the presence of ristocetin. At different times during the tryptic digestion of ^{125}I-VWF, samples were taken from the incubation mixture and incubated with washed platelets in the

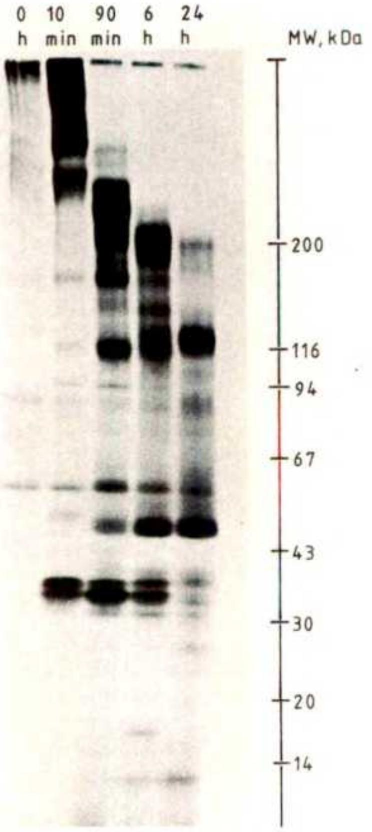

Fig 2. Degradation of ^{125}I-von Willebrand factor (VWF) by trypsin. ^{125}I-VWF was digested with trypsin for 24 hours at 37 °C. Before (0 hours) and 10 minutes, 90 minutes, 6 hours, and 24 hours after the addition of trypsin, samples were collected from the incubation mixture, added to inhibitors, and electrophoresed on a 3% to 30% polyacrylamide gradient gel. Autoradiogram of the gel is shown.

presence of ristocetin. The bound radioactivity was eluted from the platelet pellet and analyzed on 3% to 30% polyacrylamide gradient gels. Comparison of such fragments with the degradation pattern in Fig 2 showed that the fragments that became bound to the platelets had a lower mol wt than did the corresponding starting material. At ten minutes, the starting material predominantly showed fragments with mol wts >200 kd, whereas the bound material showed fragments of <200 kd. At 24 hours, a single fragment of 50 kd bound to the platelets (Fig 3A).

We have previously demonstrated that CLB-RAg 35, a monoclonal antibody that inhibits the ristocetin-induced binding of VWF to platelets, immunoprecipitated a fragment of 116 kd after 24 hours of digestion.[12] After reduction, the epitope for CLB-RAg 35 was confined to a 52- to 56-kd fragment, close to the 50-kd fragment identified here, suggesting that the tryptic fragments bound to the platelets became reduced, probably by endogenous reducing substances from the platelets.

To avoid reduction, we performed the incubation with platelets in the presence of 10 mmol NEM. The bound fragments were again eluted from the platelets and analyzed on a polyacrylamide gradient gel. The autoradiogram in Fig

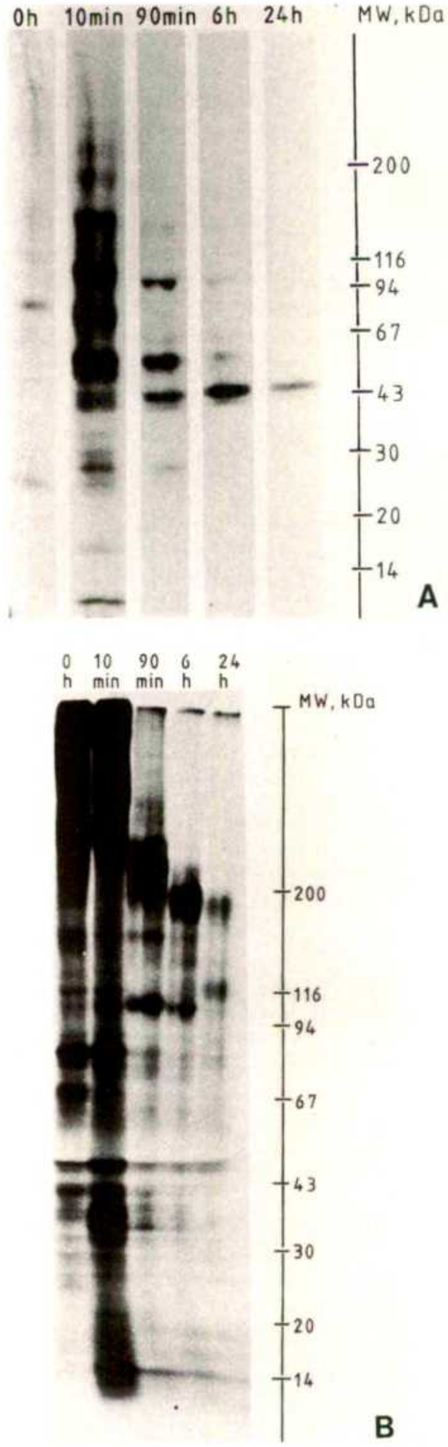

Fig 3. Analysis of tryptic fragments of ^{125}I-von Willebrand factor (VWF) bound to platelets in the presence of ristocetin. ^{125}I-VWF was digested with trypsin and at different times, samples were incubated with washed platelets and ristocetin. The bound radioactivity was eluted by resuspension of the platelets in buffer and precipitated with trichloroacetic acid (TCA). The TCA pellet was solubilized in sample buffer and applied to a 3% to 30% polyacrylamide gradient gel. The autoradiogram is shown. (A) Incubation of tryptic fragments with platelets and ristocetin without *N*-ethylmaleimide (NEM). (B) Incubation of tryptic fragments with platelets and ristocetin in the presence of 10 mmol/L of NEM.

3B shows that in this case reduction was prevented; the fragments were of a size similar to that of the starting material. The fragments that became directly bound to platelets in the presence of ristocetin showed predominance of a band at 180 and 116 kd after six-hour digestion and of 180-kd, 116-kd, and some 48-kd material after 24-hour digestion. The predominant 116-kd material bound to platelets immunoprecipitated with CLB-RAg 35 (Fig 4).

Tryptic fragments of ^{125}I-VWF binding to collagen. At different times of digestion, samples were also incubated with fibrils of collagen type III. After centrifugation, the bound radioactivity was eluted from the collagen fibrils with SDS and analyzed on a 3% to 30% polyacrylamide gradient gel. At ten minutes, only high mol wt fragments were bound to collagen. At 90 minutes, high mol wt fragments (>180 kd) and a 48-kd fragment were identified (not shown). At six hours of digestion, a single fragment of 48 kd bound to collagen (Fig 4). This fragment was recognized by immunoprecipitation with CLB-RAg 201, a monoclonal antibody that inhibits the binding of VWF to collagen.[12] A similar fragment bound to fibrils of collagen type I (not shown).

DISCUSSION

In a previous study, we identified a 116-kd tryptic fragment of VWF that contained the platelet binding domain and a 48-kd fragment that contained the collagen binding domain.[12] These tryptic fragments were immunoprecipitated by the monoclonal antibody CLB-RAg 35 that inhibits the ristocetin-induced binding of VWF to platelets, and the monoclonal antibody CLB-RAg 201, that inhibits the binding of VWF to collagen. Thus, the 116-kd and 48-kd fragments contain the epitopes for either CLB-RAg 35 or CLB-RAg 201. However, because of steric hindrance, the actual binding domains may be different from these epitopes. The binding domains may even reside on different fragments. To prove that the previously identified fragments contain the proper binding domains, we investigated the direct binding of tryptic fragments of ^{125}I-VWF to platelets in the presence of ristocetin and to collagen.

There is a preferential binding of the larger multimers of VWF to platelets in the presence of ristocetin[16,17] and to collagen.[18,19] Disruption of the VWF structure by limited proteolytic digestion will therefore diminish the binding affinity. As shown in Fig 1, there was a rapid and parallel decrease in the binding of VWF to platelets and to collagen after the onset of tryptic digestion. After 90 minutes of digestion, the decrease stabilized, but the residual binding still remained above the control value. The rapid decrease in binding to platelets in the presence of ristocetin and the low residual binding is in agreement with the study of Martin et al.[11] In that study, the ristocetin cofactor activity decreased to 25% of the starting value after ten minutes of tryptic digestion, and the residual activity was only 4% after 90 minutes.

To analyze which fragments were bound to platelets, the bound radioactivity was eluted and electrophoresed on polyacrylamide gradient gels by resuspension of the platelet pellet in buffer.[16] Analysis of the bound fragments revealed that these were substantially smaller as compared with the starting material, probably because of reduction. The 50-kd fragment which bound after 24 hours of digestion was similar to the one obtained after reduction of the 116-kd fragment immunoprecipitated by CLB-RAg 35.[12] This reduction of the bound fragments may be caused by endogenous reducing substances from the platelets, such as reduced glutathione. Reduction was prevented by adding 10 mmol/L of NEM to the incubation mixture, which had no effect on the binding itself. Another possibility, which may explain why smaller fragments were found bound to platelets than were present in the corresponding starting material, is the action of calcium-

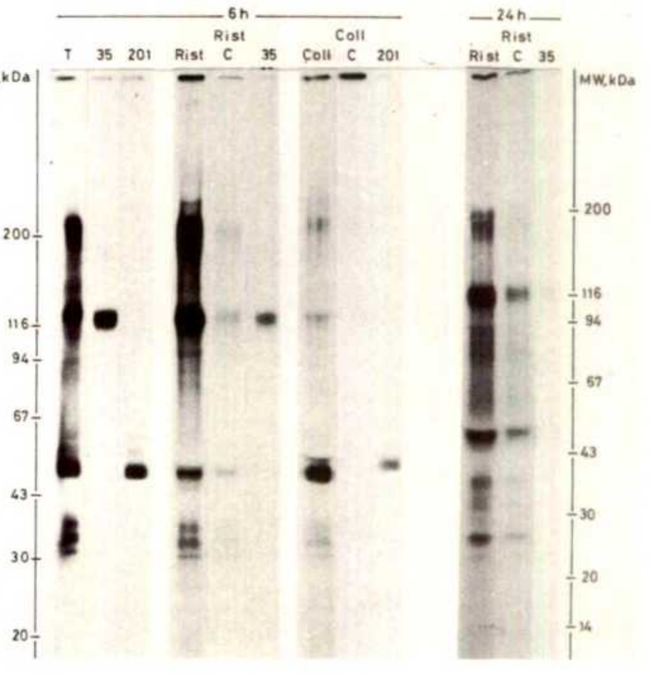

Fig 4. Immunorecognition of tryptic fragments. Autoradiogram of a 3% to 30% sodium dodecyl sulfate polyacrylamide gel. Digestion was for six hours or 24 hours as indicated. The first three panels show the total digest (T) and the material immunoprecipitated by CLB-RAg 35 and 201 (35,201). The panels marked Rist show first the material bound to platelets in the presence of ristocetin (rist) or the control platelets (c) and then the material immunoprecipitated by CLB-RAg 35. The panels marked Coll show the material bound to collagen type III fibrils (coll) and the control pellet (c) and then the material immunoprecipitated by CLB-RAg 201. The platelet binding studies were performed in the presence of 10 mmol/L of *N*-ethylmaleimide.

activated protease. The cleavage of VWF by this protease has recently been reported.[20] Calcium-activated protease of platelets is sulfhydryl-dependent and is inhibited by NEM.

In the presence of NEM, the bound fragments were of a size similar to that of the starting material. After ten minutes, high mol wt material and a 36- to 34-kd fragment were present. After 90 minutes and 6 hours, 180-kd and 116-kd fragments and fragments >180-kd were bound. After 24 hours of digestion, some 180-kd and 48-kd fragments were bound, but the prominent band was the 116-kd band. This 116-kd fragment, bound to platelets in the presence of ristocetin, was specifically immunoprecipitated by CLB-RAg 35 and is similar to the 116-kd tryptic fragment described by Martin et al,[11] which contained some residual ristocetin cofactor activity. From these combined observations, we may conclude that the domain on VWF involved in the ristocetin-induced binding to platelets resides on a 116-kd tryptic fragment.

The fragments bound to fibrils of collagen type III were eluted with SDS and analyzed on polyacrylamide gradient gels. The bound tryptic fragments were similar to those previously identified by CLB-RAg 201.[12] After ten minutes of digestion, only high mol wt material was bound. After 90 minutes, a 48-kd fragment was bound in addition to high mol wt material. After six hours, a single fragment of 48 kd was bound to collagen. From these observations, we conclude that this 48-kd tryptic fragment contains not only the epitope for CLB-RAg 201, but also the collagen binding domain.

From electron microscopic and spectroscopic studies, it is clear that the VWF molecule is a long flexible filament[21] with discrete ordered conformational domains linked by regions of random polypeptide chain.[22] Limited proteolysis offers the opportunity to identify different domains, as demonstrated here and in previous studies.[11,12] Using functional binding assays, we identified the domain involved in ristocetin-induced binding to platelets and the collagen binding domain, which were located on distinct tryptic fragments of 116-kd and 48 kd, respectively. This is in agreement with the data previously obtained with the use of monoclonal antibodies,[12] thereby demonstrating the usefulness of these antibodies in mapping the domain structure. The exact localization of the domains remains to be determined. Studies using limited proteolysis with different enzymes and identification of overlapping fragments, as well as protein sequencing and cloning studies, are currently in progress in different groups and will solve this issue in the future.

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REFERENCES

1. Hoyer LW: The factor VIII complex: Structure and function. Blood 58:1, 1981

2. Counts RB, Paskell SL, Elgee SK: Disulfide bonds and the quaternary structure of factor VIII/von Willebrand factor. J Clin Invest 62:702, 1978

3. Meyer D, Obert B, Pietu G, Lavergne JM, Zimmerman TS: Multimeric structure of factor VIII/von Willebrand factor in von Willebrand's disease. J Lab Clin Med 95:590, 1980

4. Ruggeri ZM, Zimmerman TS: The complex multimeric composition of factor VIII–von Willebrand factor. Blood 57:1140, 1981

5. Weiss HJ, Turitto VT, Baumgartner HR: Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. J Lab Clin Med 92:750, 1978

6. Houdijk WPM, Sakariassen KS, Nievelstein PFEM, Sixma JJ: Role of factor VIII–von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J Clin Invest 75:531, 1985

7. Sakariassen KS, Bolhuis PA, Sixma JJ: Human blood platelet adhesion to artery subendothelium is mediated by factor VIII–von Willebrand factor bound to the subendothelium. Nature 279:635, 1979

8. Coller BS, Peerschke EI, Scudder LE, Sullivan CA: Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: Additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. Blood 61:99, 1983

9. Ruan C, Tobelem G, McMichael AJ, Dronet L, Legrand Y, Degos L, Kieffer N, Lee H, Caen JP: Monoclonal antibody to human glycoprotein I. II. Effects on human platelet function. Br J Haematol 49:511, 1981

10. Stel HV, Sakariassen KS, Scholte BJ, Veerman ECI, van der Kwast ThH, de Groot PhG, Sixma JJ, van Mourik JA: Characterization of 25 monoclonal antibodies to factor VIII–von Willebrand factor: Relationship between ristocetin-induced platelet aggregation and platelet adherence to subendothelium. Blood 63:1408, 1984

11. Martin SE, Marder VJ, Francis CW, Loftus LS, Barlow GH: Enzymatic degradation of the factor VIII–von Willebrand protein: A unique tryptic fragment with ristocetin cofactor activity. Blood 55:848, 1980

12. Sixma JJ, Sakariassen KS, Stel HV, Houdijk WPM, In der Maur DW, Hamer RJ, de Groot PG, van Mourik JA: Functional domains on von Willebrand factor. Recognition of discrete tryptic fragments by monoclonal antibodies that inhibit interaction of von Willebrand factor with platelets and with collagen. J Clin Invest 74:736, 1984

13. Van Mourik JA, Mochtar IA: Purification of human antihemophilic factor (factor VIII) by gel-chromatography. Biochim Biophys Acta 221:677, 1970

14. Fraker PJ, Speck JC: Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3α, 6α-diphenyl glucoluril. Biochem Biophys Res Commun 80:849, 1978

15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

16. Doucet-de Bruine MHM, Sixma JJ, Over J, Beeser-Visser NH: Heterogeneity of human factor VIII. II. Characterization of forms of factor VIII binding to platelets in the presence of ristocetin. J Lab Clin Med 92:96, 1978

17. Gralnick HR, Williams SB, Morisato DK: Effect of the multimeric structure of the factor VIII/von Willebrand factor protein on binding to platelets. Blood 58:387, 1981

18. Santoro SA: Preferential binding of high molecular weight forms of von Willebrand factor to fibrillar collagen. Biochim Biophys Acta 756:123, 1983

19. Kessler CM, Floyd CM, Rick ME, Krizek DM, Lee SL, Gralnick HR: Collagen-factor VIII/von Willebrand factor protein interaction. Blood 63:1291, 1984

20. Kunicki TJ, Montgomery RR, Schullek J: Cleavage of human von Willebrand factor by platelet calcium-activated protease. Blood 65:352, 1985

21. Ohmori K, Fretto LJ, Harrison RL, Switzer MEP, Erickson HP, McKee PA: Electron microscopy of human factor VIII/von Willebrand glycoprotein: Effect of reducing reagents on structure and function. J Cell Biol 95:632, 1982

22. Loscalzo J, Handin RI: Conformational domains and structural transitions of human von Willebrand protein. Biochemistry 23:3880, 1984

CONCISE REPORT

Analysis of the Peptide Subunits of Human Neutrophil Myeloperoxidase

By William M. Nauseef and Harry L. Malech

Myeloperoxidase is a cationic protein present in the azurophilic granules of human neutrophils and constitutes 2% to 5% of neutrophil protein by weight. Despite extensive characterization of the functional importance of myeloperoxidase in the microbicidal activity of neutrophils, there is no consensus regarding the subunit composition of this enzyme or the presence of genetic polymorphism. Using isoelectric focussing and peptide mapping of chymotryptic digests of myeloperoxidase subunits under reducing and nonreducing conditions, we found two different heavy chain peptides that have overlapping but distinctive chymotryptic digest maps. One of these heavy chains has a reducible intrapeptide disulfide bond not found in the other. These results suggest that the model proposed for the structure of myeloperoxidase as a symmetric molecule with two identical heavy-light protomers may not be correct.

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MYELOPEROXIDASE (MPO) is a heme enzyme present in the azurophilic granules of human polymorphonuclear neutrophils (PMNs) and is important in the oxygen-dependent microbicidal activity of PMNs.[1-3] We have noted, as have others,[4-8] that MPO has major subunits of 59-kDa and 13.5-kDa. Electrophoretic analysis of MPO under nonreducing conditions demonstrated that some of the 59-kDa peptide migrated with an apparent molecular weight of 39-kDa, suggesting that the heavy chain of MPO might be heterogeneous. In this study we used isoelectric focussing and proteolytic peptide mapping to address the questions of isozymic variation of MPO and the structural homology of these subunit peptides. Such information is essential for the formulation of hypotheses regarding the structure of the native MPO molecule and the molecular basis of MPO deficiency, a common hereditary defect of human PMNs.[9]

MATERALS AND METHODS

Isolation of PMNs. PMNs were isolated from heparinized venous blood by differential centrifugation in Ficoll-Hypaque and sedimentation in 3% dextran as previously described.[10] After hypotonic lysis of erythrocytes, cell suspensions contained 95% to 97% PMNs.

Purification of MPO. Isolated PMNs were treated with 1 mmol/L diisopropylfluorophosphate (DFP) prior to nitrogen bomb cavitation using a modification of a technique described by Klempner et al.[11] Azurophilic granules were isolated by differential centrifugation in a discontinuous Percoll gradient[12] and were disrupted by freeze-thawing. MPO was purified as described previously.[13]

MPO antiserum. Rabbit antiserum to MPO was produced as previously described[13] and formed a single immunoprecipitin band with partially purified MPO in an Ouchterlony immunodiffusion assay. In immunoautoradiographic studies, the whole antiserum was used at a 1:40 dilution in 3% bovine serum albumin in Tris-saline buffer. In a hemagglutination inhibition assay the antiserum was active at a dilution of 1:4,096.

Nonequilibrium pH gel electrophoresis (NEPHGE). NEPHGE was performed according to the method described by O'Farrell.[14] Electrophoresis was carried out at a constant voltage of 500 V for different periods of time.

Polyacrylamide gel electrophoresis in SDS (SDS-PAGE). Samples were dissolved in solubilizing buffer (6% SDS, 4M urea, 125 mmol/L Tris 4 mmol/L EDTA, pH 6.9) with 10% 2-mercaptoethanol and a small amount of bromophenol blue (as tracking dye), and heated at 90 °C for 2 minutes prior to electrophoresis into polyacrylamide slab gels. The gels were prepared with a 3.5% stacking gel according to the method of Laemmli[15] but containing 0.2% SDS. When whole PMNs were used, 10 μg of RNase/DNase and 20% Triton X-100 were added to the sample prior to solubilization. Samples were electrophoresed toward the anode at 30 V for 18 hours constant voltage after which time the voltage was increased to 100 V until the dye front reached 10.5 cm from the origin.

Two dimensional gels (2D-PAGE). The tube gel from NEPHGE was soaked 45 minutes at room temperature in solubilizing buffer with 10% 2-mercaptoethanol and then placed at the top of the slab gel and electrophoresed as described above. Proteins in polyacrylamide gels were identified by staining the gels with R-250 Coomassie blue.

Immunoautoradiography. After separation of peptides by 2D-PAGE, the proteins were electroblotted from the gel to nitrocellulose paper according to the technique of Towbin et al.[16] The nitrocellulose paper was processed using rabbit anti-MPO and [^{125}I]-Protein A as described previously.[13] For quantitative analysis, known amounts of MPO were electroblotted and areas of nitrocellulose paper identified as MPO subunits by autoradiography were cut out and counted.

Peptide mapping. Peptide mapping was done as previously described.[17] Peptides separated by PAGE were cut from the gel and washed sequentially in 25% isopropanol and 10% methanol before being dried. Proteins in the dried gel slices were iodinated using chloramine T (100 to 300 μCi/slice), and the labelled gel pieces were dialyzed extensively against 10% methanol and lyophilized. Dried slices were digested with chymotrypsin (50 μg) at 37 °C in 50 mmol/L ammonium bicarbonate (pH 8) for 24 hours. The supernates were lyophilized and dissolved in electrophoresis buffer (15% acetic acid, 4.4% formic acid in water) prior to two-dimensional separation (electrophoresis-X chromatography). Samples were electrophoresed on cellulose-coated thin layer chromatograms (Chromagram Sheet, Cat. #13255, Eastman, Rochester, NY) in the first dimension in acetic acid/formic acid/water at 1,000 V for 9.5 cm

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using basic fuchsin as the tracking dye. This was followed by liquid chromatography in the second dimension in butanol-pyridine-acetic acid-water (13:10:2:8) with 7% 2,5-diphenyloxazole. Autoradiography of the dried sheets was then performed.

RESULTS

Unreduced purified MPO migrated in SDS-PAGE as 59-kDa, 39-kDa, and 13.5-kDa subunit peptides. Reduction of purified MPO with 10% 2-mercaptoethanol prior to electrophoresis resulted in a change in the relative amounts of 39-kDa peptide and 59-kDa peptide. The results of quantitative immunoautoradiography of varied amounts of MPO (4.4 μg to 17.2 μg) are shown in Fig 1. At all four concentrations of MPO, the relative proportion of the subunits was constant and the normalized counts are shown in Table 1. In the absence of reducing agent, the 59-kDa:39-kDa ratio was approximately 1:1 (0.91 ± 0.04), whereas in the presence of 2-mercaptoethanol it was approximately 10:1 (9.93 ± 0.3).

Two-dimensional electrophoresis (NEPHGE × SDS-PAGE) was used to estimate the pI's of these peptides. When the NEPHGE was run 1,100 volt-hours (V-h), the 13.5-kDa subunit migrated to pH 7.2 whereas the 59-kDa subunit migrated to pH 8.3 (Fig 2). Increasing the duration of NEPHGE to 2,000 V-h had no effect on the migration of the 13.5-kDa peptide, suggesting that the pI of this subunit was 7.2. After prolonged NEPHGE, the 59-kDa peptide migrated to pH 9.2, the upper limit of the established pH gradient, and therefore may not yet have reached its isoelectric pH. Of note is that in two-dimensional PAGE run under nonreducing conditions the 59-kDa and 39-kDa peptides were found at the identical pH, suggesting that under these conditions the two subunits were similarly charged (data not shown).

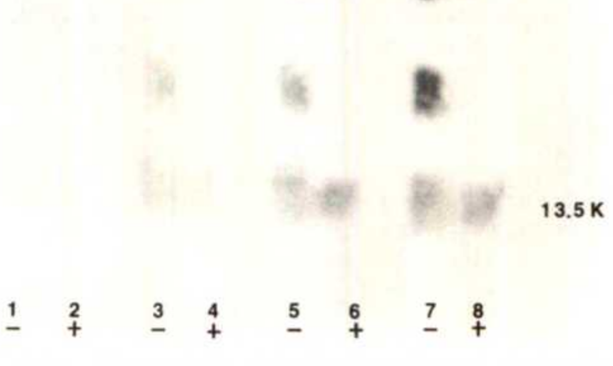

Fig 1. Quantitative immunoautoradiography of purified MPO with (+) and without (−) reduction with 2-mercaptoethanol. Purified MPO (4.4 μg, 8.8 μg, 13.2 μg, and 17.6 μg, respectively) was electrophoresed into a 13% acrylamide slab gel and electroblotted to nitrocellulose paper. After autoradiography, areas of nitrocellulose paper that correspond to MPO subunits (59-kDa, 39-kDa, 24-kDa, 13.5-kDa) were cut out and counted. The total counts in each lane were linearly related to the amount of MPO electrophoresed. After reduction most of the 39-kDa subunit is converted to 59-kDa.

Table 1. Normalized Counts of MPO Subunits From the Electroblot of Varied Amounts of Purified MPO

	Amount of MPO							
	4.4 μg		8.8 μg		13.2 μg		17.6 μg	
	Presence of 2-ME*							
Subunit	+	−	+	−	+	−	+	−
59-kDa	.72	.23	.78	.29	.75	.21	.71	.22
39-kDa	.07	.25	.03	.23	.08	.22	.07	.29
24-kDa†	.08	.24	.05	.25	.04	.29	.06	.28
13.5-kDa	.13	.28	.14	.23	.13	.28	.17	.21

*2-mercaptoethanol.

†a dimer of 3.5-kDa subunit determined by maps of chymotryptic digest.

In order to clarify the interrelationships of the 59-kDa, 39-kDa, and 13.5-kDa peptides, we compared the peptide maps of chymotryptic digests of each of the species, both in the reduced and in the nonreduced states (Fig 3). Whether reduced or unreduced the 59-kDa peptide had the same map (Fig 3, panel A). The large amount of 39-kDa peptide present in the unreduced gel and the remnant of the 39-kDa peptide left after reduction had identical maps (Fig 3, panel B). The 13.5-kDa peptide also had a similar map, whether from the reduced or unreduced sample (Fig 3, panel C). The maps of the 39-kDa and 59-kDa peptides overlapped to a great extent, although there were two major proteolytic pieces missing from the 39-kDa peptide that were prominent in the 59-kDa peptide (Fig 3, dotted circles in panel B). In contrast, the map of the 13.5-kDa peptide was markedly dissimilar from those of both the 59-kDa and 39-kDa peptides. The 24-kDa material had a map identical to that of 13.5-kDa subunit and represents a dimer of the 13.5-kDa subunit (not shown).

DISCUSSION

MPO is a heme enzyme important in PMN microbicidal activity, yet its structure is incompletely characterized, including definition of the subunits and a working model for the structure of native MPO. Models proposed include two

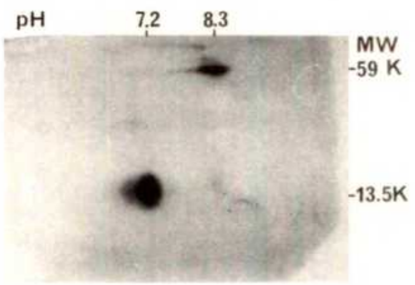

Fig 2. Immunoautoradiograph of an electroblot of two-dimensional SDS-PAGE of purified MPO. MPO was first subjected to NEPHGE in a tube gel for 1100 V-h, soaked in SDS buffer with 10% 2-mercaptoethanol, and electrophoresed into a 13% polyacrylamide slab gel. The slab gel was then electroblotted to nitrocellulose paper and processed with anti-MPO and autoradiography. The pH gradient was determined by measuring the pH of 25 mm segments of the NEPHGE run in parallel with the sample gel. The 13.5-kDa peptide is focused at pH 7.2 while the 59-kDa peptide migrated to pH 8.3.

heavy and two light chains combined as a pair of identical heavy-light protomers[4-7,18,22]; a pair of identical subunits joined by noncovalent bonds[20,23,24]; two subunits each with different molecular weight[21]; and even multiple subunits of 62-kDa, 54-kDa, 38-kDa, and 14-kDa.[7] The current model is that MPO consists of two heavy-light protomers each with a heavy subunit of approximately 59-kDa and a light subunit of approximately 14-kDa,[4-8,13] with the two protomers linked along their long axis by a single disulfide bond joining the two heavy subunits[5] or the two light subunits.[6]

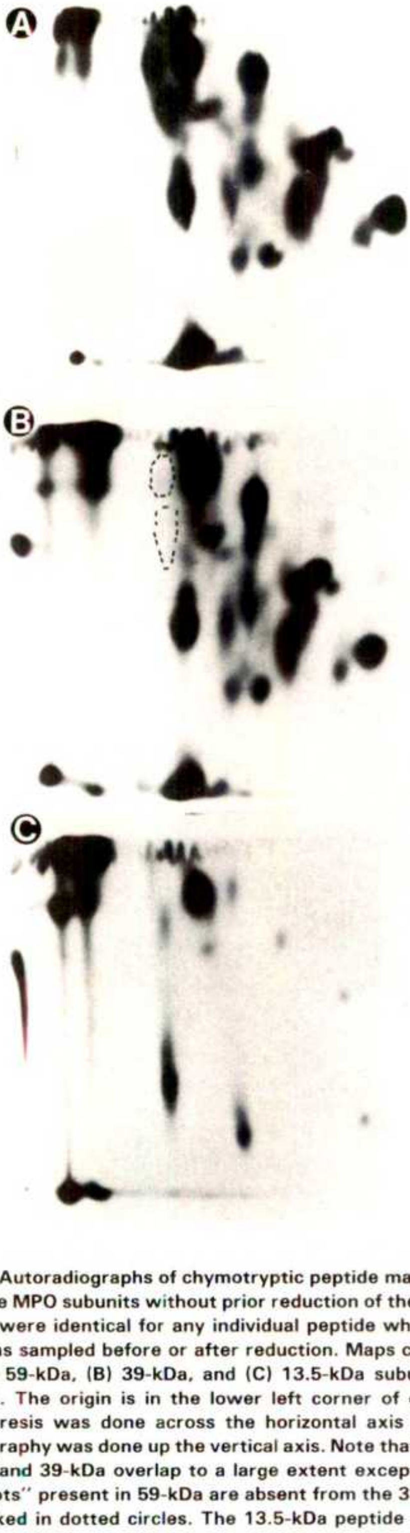

Fig 3. Autoradiographs of chymotryptic peptide maps of each of the three MPO subunits without prior reduction of the subunits. The maps were identical for any individual peptide whether that peptide was sampled before or after reduction. Maps correspond to the (A) 59-kDa, (B) 39-kDa, and (C) 13.5-kDa subunits after SDS-PAGE. The origin is in the lower left corner of each map. Electrophoresis was done across the horizontal axis and liquid chromatography was done up the vertical axis. Note that the maps of 59-kDa and 39-kDa overlap to a large extent except that two major "spots" present in 59-kDa are absent from the 39-kDa, the areas marked in dotted circles. The 13.5-kDa peptide resembles neither the 59-kDa nor the 39-kDa subunits.

The subunit structure of MPO has been the subject of considerable study but with no consensus. Using carboxymethyl-cellulose ion-exchange column chromatography, Pember et al[25] identified three forms of MPO from a single donor that were distinguished by solubility in detergent or high ionic strength solutions and by differential specific activity with various substrates. The subunit structure of the various species was different. Although all three had an 11.5-kDa subunit when reduced MPO was examined in SDS-PAGE, one form had a heavy subunit of 63-kDa whereas the other two had subunits of 57.5-kDa and 57-kDa, respectively. Although the interrelationship of the various subunits was not defined, that study suggested that there is some structural heterogeneity in human MPO. More recently Olsen and Little[26] have reported that the subunit pattern varied with the sample preparation prior to analysis. When reduced prior to heating, purified MPO in SDS-PAGE separated into two subunits (57-kDa and 10.5-kDa). Reduction of MPO concomitant with or after heating resulted in three subunits (57, 39, and 10.5) whereas heating without reduction produced only 39-kDa subunit. The amino acid composition of the separated subunits, albeit similar, were not identical and, in contrast to the studies reported here, the 39-kDa and 57-kDa species were not interconvertible.

We approximated the isoelectric points of the subunits of purified MPO by electroblotting two dimensional gels, using for the first dimension NEPHGE, a procedure with which a given peptide may not reach its isoelectric point.[14] We found no evidence for isozymic variants of the 13.5-kDa subunit of MPO from numerous subjects (data not shown). However, because the 59-kDa subunit may not have been focused to equilibrium, it cannot be stated with certainty that variants with slightly different pI's are not present.

Peptide mapping was performed in order to clarify the interrelationship of the 59-kDa and 39-kDa peptides. Based on the observed differences in the maps of these peptides, it appears that the 39-kDa and 59-kDa peptides are related but not identical and that the structure of native MPO is asymmetric with regard to the heavy chains (ie, $HH'L_2$ rather than H_2L_2). Alternatively, there may be two or three species of native MPO (ie, H_2L_2, $HH'L_2$, and H'_2L_2). In either case, since the 39-kDa peptide can be converted to a species that has an apparent molecular weight of 59-kDa after the reduction and SDS-PAGE, the 39-kDa peptide is not a proteolytic product of the 59-kDa peptide.

The data presented here suggest significant differences between the structure of the 39-kDa and 59-kDa subunits of MPO. Such differences may have functional significance, for example, as regards the location of the heme group. MPO contains two heme groups per molecule and a number of studies suggest that the two heme groups are not equivalent.[18,19] The effect of such inequivalence on peroxidative activity of native MPO is not clear. Andrews and Krinsky have reported generation of "hemi-myeloperoxidase" and found no spectral change in the heme environment nor loss of peroxidative activity in comparison to the native enzyme.[5] This suggests the heme-heme interaction in native MPO may not be critical to peroxidative activity. Similarly such differences may provide a site for subtle isozymic variation in the structure of the heavy subunit. Characterization of these differences may resolve the question of isoenzymes of MPO and may also provide insight into biosynthesis of normal MPO. Such understanding will also be fundamental to elucidating the genetic defect in hereditary MPO deficiency.

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REFERENCES

1. Klebanoff SJ: Iodination of bacteria: A bactericidal mechanism. J Exp Med 126:1063, 1967

2. Klebanoff SJ. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J Bacteriol 95:2131, 1968

3. Klebanoff SJ: A peroxidase-mediated antimicrobial system in leukocytes. J Clin Invest 46:1078, 1967

4. Andersen M, Atkin CL, Eyre HJ: Intact form of myeloperoxidase from normal human neutrophils. Arch Biochem Biophys 214:273, 1982

5. Andrews PC, Krinsky NI: The reductive cleavage of myeloperoxidase in half, producing enzymatically active hemi-myeloperoxidase. J Biol Chem 256:4211, 1981

6. Harrison JE, Pabalan S, Schultz J: The subunit structure of crystalline canine myeloperoxidase. Biochim Biophys Acta 493:247, 1977

7. Olsson I, Olofsson T, Odeberg H: Myeloperoxidase-mediated iodination in granulocytes. Scand J Haematol 9:483, 1972

8. Yamada M, Mori M, Sugimura T: Purification and characterization of small molecular weight myeloperoxidase from human promyelocytic leukemia HL-60 cells. Biochemistry 20:766, 1981

9. Parry MF, Root RK, Metcalf JA, Delaney KK, Kaplow LS, Richar WJ: Myeloperoxidase deficiency. Prevalence and clinical significance. Ann Int Med 95:293, 1981

10. Root RK, Rosenthal AS, Balestra DJ: Abnormal bactericidal, metabolic and lysosomal functions of Chediak-Higushi syndrome leukocytes. J Clin Invest 51:649, 1972

11. Klempner MS, Mikkelsen RB, Corfmann DH, Andre-Schwartz J: Neutrophil plasma membranes. I. High yield purification of human neutrophil plasma membrane vesicles by nitrogen cavitation and differential centrifugation. J Cell Biol 86:21, 1980

12. Borregaard N, Heiple JM, Simons ER, Clark RA: Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: Translocation during activation. J Cell Biol 97:52, 1983

13. Nauseef WM, Root RK, Malech HL: Biochemical and immunologic analysis of hereditary myeloperoxidase deficiency. J Clin Invest 71:1297, 1983

14. O'Farrell PZ, Goodman HM, O'Farrell PH: High regulation two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133, 1977

15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

16. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979

17. Malech HL, Marchesi VT: Hollow cylinder protein in the cytoplasm of human erythrocytes. Biochim Biophys Acta 670:385, 1981

18. Harrison JE, Schultz J: Myeloperoxidase: Confirmation and nature of heme-binding inequivalence. Biochim Biophys Acta 536:341, 1978

19. Odajima T, Yamazaki I: Myeloperoxidase of the leukocyte of normal blood. Biochim Biophys Acta 284:360, 1972

20. Matheson NR, Wong PS, Travis J: Isolation and properties of human neutrophil myeloperoxidase. Biochemistry 20:325, 1981

21. Bakkenist ARJ, Wever R, Vulsma T, Plat H, Van Gelder BF: Isolation procedure and some properties of myeloperoxidase from human leukocytes. Biochim Biophys Acta 524:45, 1978

22. Kokriakov VN, Borisov AI, Slepenkov SV, Lyzlova SN: Comparison of various physio-chemical properties of pig and cattle myeloperoxidase. Biokhimiia 47:100, 1982

23. Feldberg NT, Putterman GJ, Schultz J: Myeloperoxidase. Biochem Biophys Res Comm 37:213, 1969

24. Feldberg NT, Schultz J: Evidence that myeloperoxidase is composed of isoenzymes. Arch Biochem Biophys 148:407, 1972

25. Pember SO, Shapira R, Kinkade JM: Multiple forms of myeloperoxidase from human neutrophilic granulocytes. Arch Biochem Biophys 221:391, 1983

26. Olsen RL, Little C: Studies on the subunits of human myeloperoxidase. Biochem J 222:701, 1984

CONCISE REPORT

Use of a *Bam*HI Polymorphism in the Factor IX Gene for the Determination of Hemophilia B Carrier Status

By C.W. Hay, K.A. Robertson, S.-L. Yong, A.R. Thompson, G.H. Growe, and R.T.A. MacGillivray

A *Bam*HI polymorphism has been identified in the human factor IX gene. This polymorphism, which occurs in approximately 6% of X chromosomes, has been used to determine the carrier status of a female in a family with a history of hemophilia B. This family was uninformative for the previously reported TaqI and XmnI polymorphisms in the factor IX gene.

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BLOOD clotting factor IX is a glycoprotein comprising a single polypeptide chain with a molecular weight of 54,000. On activation, the circulating zymogen is converted to a protease, factor IXa, that activates factor X and ultimately leads to the formation of an insoluble fibrin clot.[1] Deficiency of factor IX leads to hemophilia B wherein individuals experience spontaneous bleeding and/or bleeding after trauma, which can be life-threatening. Bleeding occurs at various sites, most commonly the muscles and joints, where it can result in pain, deformity, and loss of function. Hemophilia B occurs in about 1 in every 30,000 male births[2] and is inherited as an X-linked recessive trait. Although the molecular basis of most cases of hemophilia B is not known, the cloning of the factor IX gene[3,4] has facilitated these investigations. Studies to date suggest that hemophilia B results from a variety of genetic changes[5] including partial gene deletions,[6,7] point mutations in donor splice junctions,[8] and amino acid substitutions.[9]

The determination of the genetic defect in every affected family is both expensive and time consuming. However, the discovery of three restriction fragment length polymorphisms within the factor IX gene[10,11] allows the inheritance of specific alleles to be determined by linkage to these polymorphisms. This allows carrier detection and prenatal diagnosis to be carried out with a very high degree of certainty that is not attainable with tests based on blood-clotting activity.

We have used a newly identified *Bam*HI polymorphism in the factor IX gene to determine the carrier status of a female in a hemophilia-B family that was uninformative for the other polymorphisms in the factor IX gene.

MATERIALS AND METHODS

Informed consent was obtained from blood donors according to the guidelines established by the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia.

Isolation of cloned fragments of DNA. A bovine liver cDNA library constructed in pBR322[12] was screened with a heptadecadeoxyribonucleotide labeled with γ-[^{32}P] ATP using T4 polynucleotide kinase. Positive colonies were detected by autoradiography. To isolate the human factor IX gene, one million phage of the Maniatis human genomic library[13] were screened using a bovine factor IX cDNA under conditions of reduced stringency to allow hybridization of homologous sequences from different species. The identities of the bovine cDNA and human genomic clones were confirmed by DNA sequence analysis. All experiments involving recombinant DNA were performed according to the guidelines set down by the Medical Research Council of Canada.

Southern blot analysis. Aliquots of genomic DNA (10 μg) were digested with the appropriate restriction endonuclease (30 u) at 37 °C for 16 hours and the resulting fragments were separated by electrophoresis in an agarose gel in buffer containing 40 mmol/L Tris pH 8.3, 2 mmol/L EDTA, 20 mmol/L acetic acid. After denaturation, the DNA was transferred to nitrocellulose[14] and the blots were analyzed by using an appropriate subclone of the human factor IX gene as a hybridization probe. The DNA probe was labeled with either [^{32}P] α-dATP or [^{32}P] α-dCTP (Amersham) to a specific activity of 2×10^8 cpm/μg using random sequence hexadeoxynucleotides as primers (Pharmacia, Dorval, Quebec) for elongation with the Klenow fragment of DNA polymerase I.[15] Hybridization was carried out in 50% formamide at 37 °C and the filter was washed in 0.1 × SSC, 0.1% SDS at 50 °C.[16] The blot was visualised by autoradiography for 16 hours at −70 °C using Kodak XK-1 x-ray film and an intensifying screen (Du Pont, Lachine, Quebec).

Factor IX antigen and clotting activity assays. The factor IX antigen levels were assayed using two methods. The first method used a noncalcium-dependent polyclonal antibody preparation for both the coating and labeled species and the second method used a monoclonal antibody to an epitope on the heavy chain of factor IXa[17] as the solid phase and a calcium-dependent polyclonal antibody fraction (which reacted with light chain epitopes of factor IXa[18]) as the labeled species. The factor IX clotting activities were measured with a one-stage assay using factor IX deficient plasma.[19]

RESULTS AND DISCUSSION

Isolation of cloned factor IX DNA fragments. Initially, we used a heptadecadeoxyribonucleotide coding for residues 70 to 75 of the mature bovine factor IX to screen a bovine liver cDNA library.[12] A clone with a 2.5 Kbp insert was isolated and found to code for eight residues of the leader propeptide, the mature factor IX protein, and 1.2 Kbp of 3′ noncoding sequence. The bovine factor IX cDNA was then used to isolate part of the human factor IX gene (correspond-

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ing to λHIX3 of Anson et al[3]) that covered the three useful restriction fragment length polymorphisms described previously.[10,11]

Carrier detection studies. Using part of the factor IX clone as a hybridization probe, carrier detection studies were performed on a hemophilia B family living in British Columbia. The son was a hemophiliac and a positive family history confirmed that his mother was an obligate carrier of hemophilia B (Fig 1). Thus, the daughter was also at risk of being a carrier. DNA was isolated from blood samples taken from the family members and examined for the factor IX *Taq*I and *Xmn*I polymorphisms by performing Southern blot analysis.[14] Both restriction fragment length polymorphisms were uninformative as all members of the family had the *Taq*I 1.8 Kbp allele and the *Xmn*I 11.5 Kbp allele (results not shown).

Digestion of DNA samples with *Bam*HI followed by Southern blot analysis revealed a polymorphic site within the factor IX gene yielding a DNA fragment of 23 Kbp. Genomic DNA which did not contain the *Bam*HI polymorphic site produced a fragment of 25 Kbp. Southern blot analysis of the hemophilia B family was carried out after digestion of the DNA samples with *Bam*HI (Fig 1). The father had the 25 Kbp allele while the mother was heterozygous for both alleles. Since the hemophiliac son inherited the 23 Kbp allele, this allele must be associated with the hemophilia. The daughter was homozygous for the 25 Kbp allele and was thus not a carrier of hemophilia B.

As the factor IX gene *Bam*HI polymorphism had not previously been used to determine hemophilia B carrier status, the factor IX clotting activity and the factor IX antigen levels were measured for the hemophilia-B family members and for an unaffected, unrelated male who had the *Bam*HI polymorphic site (Table 1). The factor IX clotting activity and antigen levels of the daughter were in complete agreement with the diagnosis of her carrier status based on the *Bam*HI polymorphism. The antigen studies showed that the circulating factor IX in the son with hemophilia B contained epitopes present on both the light and heavy chains of factor IXa. Also, the presence of the *Bam*HI polymorphic site in the control subject affected neither factor IX clotting activity nor the factor IX antigen levels.

Table 1. Factor IX Antigen and Clotting Activity Assays

Subject	Factor IX (% of normal) IX:C	IX:Ag$_1$	IX:Ag$_2$
Father	96	105	113
Mother (carrier)	18	73	70
Son (hemophiliac)	2.6	61	63
Daughter	92	88	90
Control	108	104	121

Factor IX clotting activities (IX:C) and factor IX antigen levels (IX:Ag) of members of the hemophilia-B family and an unrelated, unaffected male with the BamHI polymorphic site. Two methods were used to determine the factor IX antigen levels (see Materials and Methods) and results were expressed as percent of normal (corresponding to 4 μg/mL citrated plasma). The son had not been transfused during the previous 9 months.

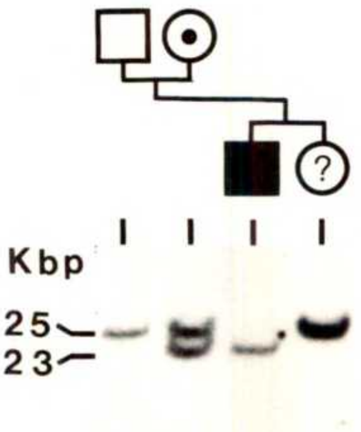

Fig 1. Carrier diagnosis of daughter in hemophilia-B family: ■ affected male; ⊙ obligate carrier. DNA samples were digested with *Bam*HI and the blot was analyzed with a 2.5 Kbp *Hind*III-*Eco*RI subclone of the human factor IX gene (equivalent to probe VIII of Giannelli et al[20]).

Location of the BamHI *polymorphism*. The location of the *Bam*HI polymorphism within the factor IX gene was determined by Southern blot analysis of genomic DNA from the affected family and from a normal control who had the 23 Kbp *Bam*HI fragment. When DNA was cut with *Bam*HI and *Bgl*II and the Southern blot analyzed with a probe from the 3′ region of the *Bam*HI 25 Kbp fragment, a single band of 5.9 Kbp was observed in all samples, suggesting that the *Bam*HI polymorphic site is not close to the *Bam*HI site in intron 6 (between exons f and g, Fig 2A). DNA was then cut with *Bam*HI and *Sph*I and analyzed by using a 5′ probe (Fig 2B). In this analysis, two hybridizing bands were observed that were 15.0 Kbp and 13.0 Kbp in size. For the affected family, the pattern of large and small bands was identical to the Southern blot of DNA cut with *Bam*HI alone (compare Fig 1 with lanes 1 to 4 of Fig 2B). In addition, the normal individual with the 23 Kbp *Bam*HI allele had the 13.0 Kbp *Bam*HI/*Sph*I fragment. From these studies, we conclude that the *Bam*HI polymorphic site is located 2 Kbp downstream of the *Bam*HI site in the 5′ flanking sequence of the factor IX gene.

Frequency of the BamHI *polymorphism.* The frequency of the *Bam*HI polymorphism in the general population was estimated by examining the DNA from 24 male and 36 female unrelated Caucasians (a total of 96 X chromosomes). The 23 Kbp *Bam*HI allele was found to occur with a frequency of 0.06 so that 11% of females should be heterozygous for the 25/23 Kbp alleles. The lack of linkage disequilibrium between the *Bam*HI polymorphic loci and the factor IX gene *Taq*I and *Dde*I polymorphic loci (CW Hay, in preparation) means that, despite its low frequency, the *Bam*HI polymorphism will be useful in carrier diagnosis in hemophilia-B families that are uninformative for other factor IX polymorphisms.

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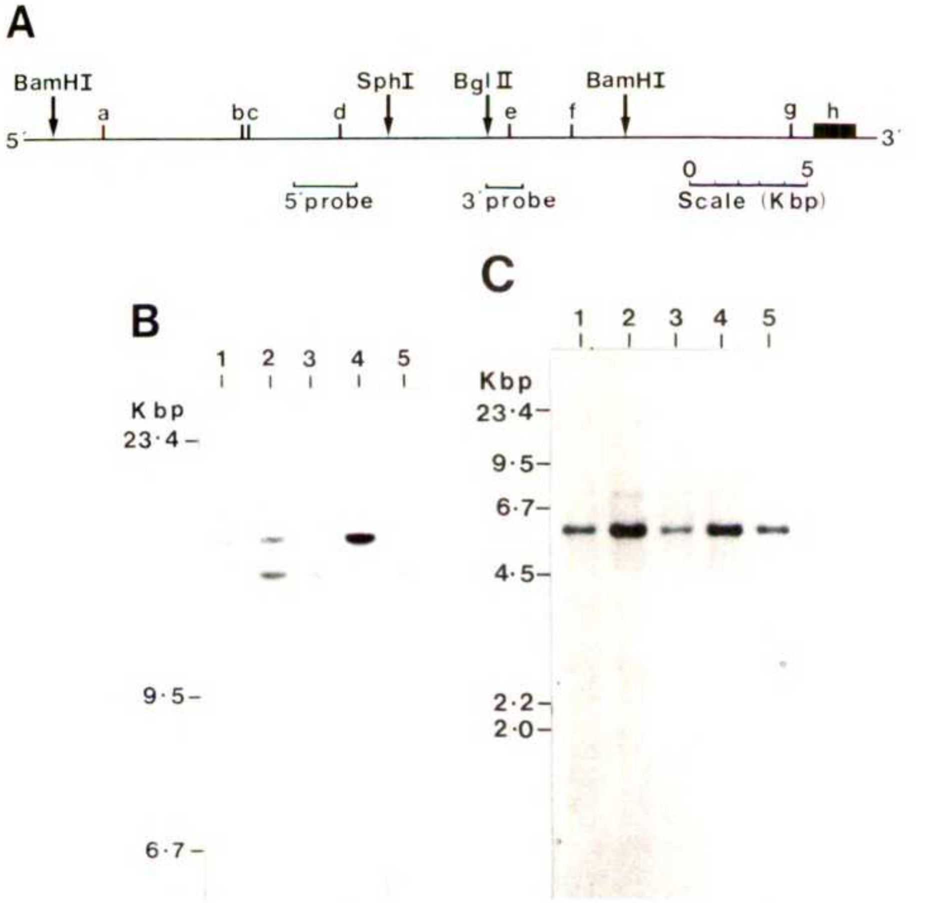

Fig 2. **Determination of the position of the *Bam*HI polymorphic site. (A) Schematic representation of the human factor IX gene[3,4] showing the position of the eight exons (a-h), the position of the relevant recognition sites for *Bam*HI, *Sph*I, and *Bgl*II,[4] and the genomic probes used in the Southern blot analyses. (B) Southern blot analysis of *Bam*HI/*Sph*I digests of genomic DNA using the 5′ probe. Lanes: 1, father; 2, mother (obligate carrier); 3, son (hemophiliac); 4, daughter; 5, unrelated, unaffected male with the *Bam*HI polymorphic site. (C) Southern blot analysis of *Bam*HI/*Bgl*II genomic digests using the 3′ probe. The lanes are in the same order as in panel B.**

REFERENCES

1. Jackson CM, Nemerson Y: Blood coagulation. Ann Rev Biochem 49:765, 1980

2. McKee PA: Hemostasis and disorders of blood coagulation, in Stanbury JB, et al (eds): The Metabolic Basis of Inherited Disease (ed 5). New York, McGraw-Hill, 1983, p 1531

3. Anson DS, Choo KH, Rees DJG, Giannelli F, Gould K, Huddleston JA, Brownlee GG: The gene structure of human anti-hemophilic factor IX. EMBO J 3:1053, 1984

4. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K: Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). Biochemistry 24:3736, 1985

5. Lawn RM: The molecular genetics of hemophilia: Blood clotting factors VIII and IX. Cell 42:405, 1985

6. Peake IR, Furlong BL, Bloom AL: Carrier detection by direct gene analysis in a family with haemophilia B (factor IX deficiency). Lancet i:242, 1984

7. Hassan HJ, Leonardi A, Guerriero R, Chelucci CM, Cianetti L, Ciavarella N, Ranieri P, Pilolli D, Peschle C: Hemophilia B with inhibitor: Molecular analysis of the subtotal deletion of the factor IX gene. Blood 66:728, 1985

8. Rees DJG, Rizza CR, Brownlee GG: Hemophilia B caused by a point mutation in a donor splice junction of the human factor IX gene. Nature 316:643, 1985

9. Noyes CM, Griffith MJ, Roberts HR, Lundblad RL: Identification of the molecular defect in factor IX Chapel Hill: Substitution of histidine for arginine at position 145. Proc Natl Acad Sci USA 80:4200, 1983

10. Camerino G, Grzeschik KH, Jaye M, De La Salle H, Tolstoshev P, Lecocq JP, Heilig R, Mandel JL: Regional localization on the human X chromosome and polymorphism of the coagulation factor IX gene (hemophilia B locus). Proc Natl Acad Sci USA 81:498, 1984

11. Winship PR, Anson DS, Rizza CR, Brownlee GG: Carrier detection in hemophilia B using two further intragenic restriction fragment length polymorphisms. Nucleic Acids Res 12:8861, 1984

12. MacGillivray RTA, Davie EW: Characterization of bovine prothrombin mRNA and its translation product. Biochemistry 23:1626, 1984

13. Lawn RM, Fritsch EF, Parker RC, Blake G, Maniatis T: The isolation and characterization of linked δ-and β-globin genes from a cloned library of human DNA. Cell 15:1157, 1978

14. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

15. Feinberg A, Vogelstein B: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6, 1983

16. Kan YW, Dozy AM: Polymorphism of DNA sequence adjacent to human β-globin structural gene: Relationship to sickle mutation. Proc Natl Acad Sci USA 75:5631, 1978

17. Thompson AR: Monoclonal antibody to an epitope on the heavy chain of factor IX missing in three hemophilia B patients. Blood 62:1027, 1983

18. Bray GL, Weinmann AF, Thompson AR: Calcium-specific immunoassays for factor IX: Reduced levels of antigen in patients with vitamin K disorders. J Lab Clin Med (in press)

19. Thompson AR: Factor IX antigen by radioimmunoassay. J Clin Invest 59:900, 1977

20. Giannelli F, Choo KH, Winship PR, Rizza CR, Anson DS, Rees DJG, Ferrari N, Brownlee GG: Characterization and use of an intragenic polymorphic marker for detection of carriers of hemophilia B (factor IX deficiency). Lancet i:239, 1984

CONCISE REPORT

Interleukin 3 Promotes the Differentiation of Isolated Single Megakaryocytes

By Toshiyuki Ishibashi and Samuel A. Burstein

Interleukin 3 (IL3) promotes the proliferation of murine hematopoietic progenitor cells, including the megakaryocytic colony-forming cell (CFU-MK). To determine whether IL3 influences more differentiated cells in the murine megakaryocytic lineage, IL3 was added to microcultures of single megakaryocytes isolated from CFU-MK-derived colonies. After two days of culture, cell diameter and DNA content were analyzed. Eighty-five percent of cells initially between 12 and 20 µm in diameter increased in size in response to IL3, while only 12% of these cells increased in size in its absence. The percentage of cells responding to IL3 varied inversely with the size of the initial cells. The culture procedure did not alter the relatively linear relationship between size and ploidy, suggesting that increments in cell size are accompanied by increased ploidy. After three days of culture, acetylcholinesterase (AchE) activity was assayed. A small but significant increment in AchE activity was observed in the presence of IL3. The data show that, at least in vitro, IL3 is a differentiation-promoting factor of the murine megakaryocytic lineage. ***© 1986 by Grune & Stratton, Inc.***

INTERLEUKIN 3 (IL3), a hematopoietic colony-stimulating factor, promotes the proliferation of multiple murine hematopoietic lineages, including hematopoietic cell lines[1]; pluripotent stem cells (CFU-S) of both limited or more extensive proliferative potential[2,3]; and more differentiated hematopoietic progenitors such as the myeloid, erythroid, mast cell, and perhaps pre-B cell.[4-8] Among the activities of IL3 is the ability to promote the proliferation of the megakaryocytic colony-forming cell (CFU-MK).[9]

Recent studies in our laboratory have shown that, in a serumless culture system, purified IL3 promotes the appearance of large polyploid megakaryocytes.[10] Acetylcholinesterase (AchE) production, a relatively specific marker of the megakaryocytic lineage in mice, is concurrently increased.[11,12] Although induction of proliferation of CFU-MK had been previously shown to be a property of IL3,[9] the positive influence of this factor on what are generally considered markers of differentiation (size, ploidy, and AchE content), suggested to us that IL3 might also promote this phase of megakaryocytopoiesis. Since previous studies had been performed using unfractionated marrow, the possibility that accessory cells may have provided differentiation-promoting activity could not be excluded. To determine whether IL3 promotes megakaryocytic differentiation directly, the present studies were performed using single megakaryocytes isolated from CFU-MK-derived clones. The advantage of this approach is that such clones contain both small, low-ploidy and large, high-ploidy cells, thereby allowing one to examine the effect of IL3 on megakaryocytes in varying stages of differentiation.

MATERIALS AND METHODS

Culture of megakaryocytic colonies. Six- to eight-week-old specific pathogen-free $C_{57}Bl/6$ male mice obtained from Jackson Laboratories, Bar Harbor, Me, were used for all experiments. Bone marrow of the femur was flushed and resuspended through an 18 followed by a 22 gauge needle. Marrow was enriched for progenitor cells on a 1.070/1.077g/cm^3 two-step Percoll gradient and cultured in 35 mm dishes at 10^4 cells/mL in Iscove's modification of Dulbecco's medium (medium) containing 1% methylcellulose, 15% horse serum, 5% pokeweed mitogen-stimulated spleen cell conditioned medium, 50 µmol/L β-mercaptoethanol (β-ME), and 100 U/mL of penicillin-streptomycin. Cells were incubated under a humidified atmosphere of 5% of CO_2 in air at 37 °C.

Single cell culture of CFU-MK-derived megakaryocytes. Megakaryocytic colonies were identified in situ as previously described.[13] The appearance of megakaryocytic colonies in methylcellulose is similar to those grown in agar gel. After five days of culture randomly chosen colonies were selectively removed under an inverted microscope with a micropipette in 2 µL volumes and dispersed into 2 mL of medium containing 50 µmol/L β-ME. Fewer than five colonies were removed at a time to maximize the dilution of the initial culture constituents. Individual cells were removed in 2 µL volumes and cultured for 48 hours in 96 microwell plate covers in 35 µL of medium containing 3% bovine serum albumin, 50 µmol/L β-ME, 1% Nutricyte (J. Brooks Laboratories, Olivenhain, Calif); a serum-free medium supplement containing albumin, transferrin, and insulin ± 5 U/mL purified IL3. The final dilution of the initial culture constituents was at least 1:3,700.

Sizing of megakaryocytes. Cell diameter was measured on the same cells before and after the culture period with an inverted microscope equipped with an ocular micrometer.[14] An increase in diameter was defined as an increment of at least 0.5 µm. Four hundred and one single megakaryocytes were examined.

Ploidy analysis of megakaryocytes. After determining the diameter, individual cells were removed from the microwells, deposited on glass slides, and fixed with 70% ethanol (ETOH) for ten minutes. The DNA was stained with 1.7×10^{-5} mol/L chromomycin A_3 containing 1 mmol/L $MgSO_4$ for 30 minutes as described previously.[15] Ploidy was measured with a fluorescence microscope equipped with a photometer. Granulocytic cells were employed as 2N standards.

Assay of AchE activity of megakaryocytes. The single cell culture procedure employed for the AchE studies was similar to that described above with the exception that no β-ME was added and that the colonies were dispersed in 2 mL of medium containing 0.5 mmol/L diisopropylfluorophosphate for 20 minutes to inactivate endogenous cholinesterase prior to deposition of single cells into

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Table 1. The Effect of IL3 on the Size of Single Megakaryocytes

Initial Cell Diameter (μm)	Estimated Ploidy (N)	Number of Cells Increasing in Diameter (%)*	
		−IL3	+IL3
12–20	<8	4/34 (12)	94/111 (85)
20–25	8–16	3/30 (10)	48/75 (64)
25–30	16–32	1/19 (5)	12/50 (24)
>30	32	0/14 (0)	3/68 (4)

*Of all cells increasing in size, the mean increment was 1.4 ± 0.9 μm in the absence of IL3 and 2.9 ± 2.0 μm in the presence of IL3. The range of increments was 0.5 to 15.7 μm.

wells.[11] After 72 hours of culture, 165 μL of a solution of 0.2% Triton X-100 in 1 mmol/L EDTA, 0.12 mol/L NaCl, and 50 mmol/L HEPES, pH 7.5 was added to each well containing a cell. Twenty μL of acethylthiocholine iodide was then added (final concentration 0.56 mmol/L). After six hours of incubation, 10 μL of 0.4 mmol/L coumarinphenylmaleimide[16] was added to 20 μL of the reaction mixture in borosilicate glass tubes, followed by the addition of 2 mL of 5 mmol/L sodium acetate (pH 5) containing 1 mmol/L EDTA and 0.2% Triton X-100. The fluorescence emission was measured with a filter fluorometer with an excitation filter of 390 nm and an emission filter of 450 nm. Cells of initial diameters of 15 to 25 μm were chosen for this assay.

RESULTS

Cell diameter. Cell diameter of 401 single megakaryocytes was measured at the outset of culture and 48 hours later. As demonstrated in Table 1, an increase in cell diameter occurred in response to IL3 addition, but this increase was dependent on the initial diameter of the examined cell. Eighty-five percent of cells initially between 12 and 20 μm in diameter increased in response to the factor. This percentage declined to 4% of cells initially greater than 30 μm in diameter. Conversely, in the absence of IL3, only 12% of cells initially between 12 and 20 μm in diameter increased in size.

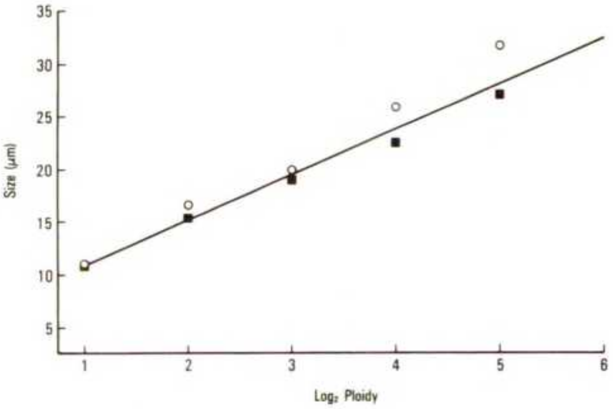

Fig 1. Composite linear regression of size and ploidy of single megakaryocytes before and after culture with IL3. The open circles represent the mean diameters before culture while the closed squares represent the mean diameters after culture in the presence of IL3. No significant differences in the relationship between size and ploidy were observed ($P > 0.05$). Thus, the ploidy of the cells at the outset of culture was estimated from the initial cell diameter.

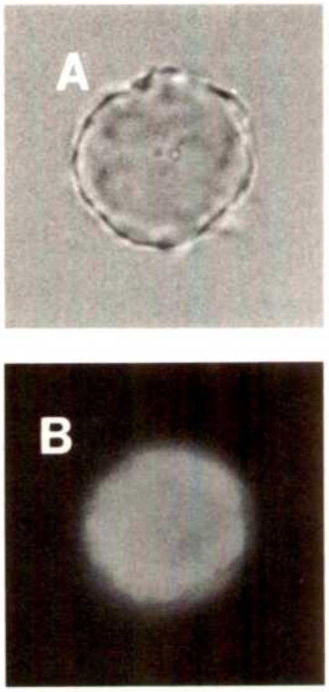

Fig 2. (A) A single megakaryocyte 48 hours after culture in the presence of 5 U/mL IL3 (×640). Initial diameter was 20.8 μm with an estimated ploidy of ≤16N. The final diameter prior to fixation was 36.5 μm. (B) The identical megakaryocyte stained with chromomycin A_3. Measured ploidy of this cell was 64N (×640).

Ploidy measurement. To determine if increments in cell size in response to IL3 were accompanied by increases in DNA content, the ploidy and size of 137 individual cells were measured prior to initiation of culture (n = 76) and after 48 hours of culture (n = 61). As shown in Fig 1, the relatively linear relationship between size and ploidy was unaffected by the culture procedure. These results indicate that the majority of the cells responding of IL3 (85% of cells 12 to 20 μm)

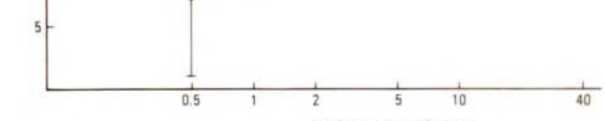

Fig 3. AchE activity of single cells as a function of IL3 concentration (mean percent increment ±1 SD). The increment in AchE activity is significant at all doses of IL3 as compared with cells cultured in its absence ($P < 0.01$; analysis of variance).

were $\leq$16N. These data also suggest that increments in cell size are likely to be associated with increments in DNA content, rather than cell spreading or isolated cytoplasmic maturation. Although cells greater than 32N were never observed, occasional cells of 64N ploidy were noted after culture with IL3 (Fig 2).

AchE Activity. AchE activity was assayed fluorometrically in cells initially 15 to 25 μm in diameter. Individual cells were cultured in microculture wells containing 35 μL defined medium with various concentrations of IL3. Figure 3 shows that a small but significant increment of this activity was observed in the presence of IL3.

DISCUSSION

These data demonstrate that IL3, a factor that promotes the proliferation of the CFU-MK, is sufficient to promote the differentiation of more distal cells in the megakaryocytic lineage as assessed by measurements of cell size, ploidy, and AchE content. Whether IL3 is necessary for the induction of megakaryocytic differentiation in vitro is unknown. The lesser influence of IL3 on large, high ploidy megakaryocytes may reflect a declining number of receptors for this factor as cells mature. Alternatively, increments in size, ploidy, or enzyme content may not be easily detectable in cells of greater than 16N ploidy and which are near the end of the maturation sequence.

The influence of IL3 on the latter stages of megakaryocytopoiesis may not be unique to this lineage. IL3 has been demonstrated to influence the expression of major histocompatibility antigens on thymus-dependent mast cells,[17] and to stimulate cell division and phagocytosis in single isolated peritoneal macrophages.[18] Another factor, the human granulocyte-macrophage colony-stimulating factor, has been shown to inhibit neutrophil migration.[19] Thus, since the range of effects of IL3 and other colony-stimulating factors may be more global than previously believed, the influence of these regulators of hematopoietic progenitor cells should also be considered in the latter differentiative stages of hematopoiesis, including, perhaps, the delivery of formed blood elements into the circulation.

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REFERENCES

1. Dexter TM, Garland J, Scott D, Scolnick E, Metcalf D: Growth of factor-dependent hemopoietic precursor cell lines. J Exp Med 152:1036, 1980
2. Rennick DM, Lee FD, Yokota T, Arai K-I, Cantor H, Nabel GJ: A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: Multiple activities of interleukin 3. J Immunol 134:910, 1985
3. Spivak JL, Smith RRL, Ihle JN: Interleukin 3 promotes the in vitro proliferation of murine pluripotent hematopoietic stem cells. J Clin Invest 76:1612, 1985
4. Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B: Biologic properties of homogenous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. J Immunol 131:282, 1983
5. Bazill GW, Haynes M, Garland J, Dexter TM: Characterization and partial purification of a haematopoietic cell growth factor in WEHI-3 cell conditioned medium. Biochem J 210:747, 1983
6. Goodman JW, Hall EA, Miller KL, Shinpock SG: Interleukin 3 promotes erythroid burst formation in "serum-free" cultures without detectable erythropoietin. Proc Natl Acad Sci USA 82:3291, 1985
7. Nabel G, Galli SJ, Dvorak AM, Dvorak HF, Cantor H: Inducer T lymphocytes synthesize a factor that stimulates proliferation of cloned mast cells. Nature 291:332, 1981
8. Palacios R, Henson G, Steinmetz M, McKearn JP: Interleukin-3 supports growth of mouse pre-B-cell clones in vitro. Nature 309:126, 1984
9. Quesenberry PJ, Ihle JN, McGrath E: The effect of interleukin 3 and GM-CSA-2 on megakaryocyte and myeloid clonal colony formation. Blood 65:214, 1985
10. Burstein SA: Interleukin 3 promotes maturation of murine megakaryocytes in vitro. Blood Cells (in press)
11. Burstein SA, Boyd CN, Dale GL: Quantitation of megakaryocytopoiesis in liquid culture by enzymatic determination of acetylcholinesterase. J Cell Physiol 122:159, 1985
12. Jackson CW: Cholinesterase as a possible marker for early cells of the megakaryocytic series. Blood 42:413, 1973
13. Burstein SA, Adamson JW, Thorning D, Harker LA: Characteristics of murine megakaryocytic colonies in vitro. Blood 54:169, 1979
14. Burstein SA, Adamson JW, Erb SK, Harker LA: Megakaryocytopoiesis in the mouse: Response to varying platelet demand. J Cell Physiol 109:333, 1981
15. Chatelain C, Burstein SA: Fluorescence cytophotometric analysis of megakaryocytic ploidy in culture: Studies of normal and thrombocytopenic mice. Blood 64:1193, 1984
16. Parvari R, Pecht I, Soreq H: A microfluoremetric assay for cholinesterases, suitable for multiple kinetic determination of picomoles of release thiocholine. Anal Biochem 133:450, 1983
17. Wong GHW, Clark-Lewis I, Hamilton JA, Schrader JW: P cell stimulating factor and glucocorticoids oppose the action of interferon-inducing Ia antigens on T-dependent mast cells (P cells). J Immunol 133:2043, 1984
18. Clark-Lewis I, Crapper RM, Leslie K, Schrader S, Schrader JW: P cell stimulating factor: Molecular and biological properties, in Sorg C, Schimpl A (eds): Cellular and Molecular Biology of Lymphokines. Orlando, Fla, Academic, 1985, p 455
19. Gasson JC, Weisbart RH, Kaufman SE, Clark SC, Hewick RM, Wong GG, Golde DW: Purified human granulocyte-macrophage colony-stimulating factor: Direct action on neutrophils. Science 226:1339, 1984

CONCISE REPORT

Mediation of Platelet Adhesion to Fibrillar Collagen in Flowing Blood by a Proteolytic Fragment of Human von Willebrand Factor

By Kjell S. Sakariassen, Edith Fressinaud, Jean-Pierre Girma, Hans R. Baumgartner, and Dominique Meyer

The effect of purified von Willebrand Factor (vWF) fragments, SpII (dimer of two 110 kd subunits) and SpIII (dimer of two 170 kd subunits) obtained with *S aureus* V-8 protease was tested upon platelet adhesion to collagen. Purified fibrillar human collagen coated onto cover slips was incubated with SpII, SpIII, or undigested vWF and exposed to reconstituted human blood in a parallel-plate perfusion chamber at a high shear rate. Platelet-collagen interactions were estimated using ^{51}Cr-platelets and quantitative morphometry. When blood was reconstituted with citrated autologous plasma, SpIII and vWF strikingly enhanced platelet adhesion to collagen whereas SpII had no effect. When blood was reconstituted with human albumin and divalent cations, SpIII and vWF again promoted platelet adhesion to collagen. In conclusion, our data suggest that (1) SpIII, the *N*-terminal portion of vWF which binds to platelet membrane glycoprotein Ib, functionally substitutes for vWF in supporting platelet adhesion to collagen; (2) SpII, the *C*-terminal portion which binds to glycoprotein IIb/IIIa, has no such effect; (3) in addition to its platelet binding domain, SpIII contains another site for binding to collagen; and (4) the multimeric structure of vWF is not required for platelet adhesion to collagen.

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VON WILLEBRAND Factor (vWF), a large multimeric glycoprotein with mol wt ranging from 1 to 15 × 10^6 daltons, is composed of dimers of two disulfide linked ∼250 kd chains. vWF plays a crucial role in normal hemostasis by mediating platelet adhesion to the subendothelium at high wall shear rates.[1] It has been shown to bind to glycoprotein Ib (GPIb) in the presence of ristocetin[2] and to glycoprotein IIb/IIIa (GPIIb/IIIa) induced by thrombin.[3] The axis "platelet GPIb-vWF-subendothelium" has been postulated as the most important to promote platelet adhesion.[4,5] It is not clear at present whether the pathway involving vWF and GPIIb/IIIa also plays a significant role in platelet-vessel wall interactions.

Both plasma and vessel wall vWF are required for platelet adhesion.[1,6] The binding of plasma vWF to subendothelium precedes platelet adherence, indicating that adhesion occurs through vessel wall bound vWF.[7] It is unknown whether the ability of vWF to interact with the platelets and the vessel wall in flowing blood resides in its quaternary conformation. Proteolytic fragments of vWF possessing binding domains for platelet membrane glycoproteins and collagen have been described.[8-10] *S aureus* V-8 protease selectively cleaves vWF, producing two complementary dimeric fragments. Fragment III (SpIII), the *N*-terminal portion of vWF subunit with apparent mol wt of 320 kd, binds to platelet membrane GPIb in the presence of ristocetin.[9-11] Fragment II (SpII), the *C*-terminal end of vWF subunit with a mol wt of 215 kd, binds to GPIIb/IIIa in the presence of thrombin.[10]

We report here results of in vitro perfusion experiments using as perfusates red cells and labeled platelets reconstituted with either citrated autologous plasma or a human albumin solution and as reactive surface human fibrillar collagen treated with purified SpII, SpIII, or undigested vWF. SpIII promoted platelet adhesion as effectively as undigested vWF, while SpII had no effect. Our data suggest that purified SpIII functionally substitutes for vWF, as it possesses the two domains required for platelet adhesion to collagen, one binding to GPIb and the other to collagen.

MATERIALS AND METHODS

vWF, SpII, and SpIII preparations. Purification of human vWF from commercial factor VIII concentrates (Hyland Division, Travenol Laboratories Inc, Los Angeles) was performed as previously described.[12,13] Purified vWF contained 193 vWF:Ag U/mg and 108 vWF:RCo U/mg and less than 0.1% contaminating fibrinogen and fibronectin. Polyacrylamide-agarose gel electrophoresis showed vWF multimers with mol wt ranging from 1 to more than 12 × 10^6 daltons.[13]

Digestion of purified vWF by *S aureus* V-8 protease (Miles Laboratories Inc, Naperville, Ill) and purification of the two main fragments SpII (Mr 215 kd) and SpIII (Mr 320 kd) were performed as recently described.[10,11] Assessment of purity was estimated by polyacrylamide gel electrophoresis[11] and by immunoradiometric assay[14] with selected monoclonal antibodies to vWF.[10] The SpII preparation contained less than 10 parts SpIII or vWF per million and the SpIII less than 20 parts SpII or vWF per million.

Preparation of human fibrillar type III collagen. Type III collagen was purified from lyophilized pepsin-extracted human placenta collagens (provided by Dr H. Sage and Dr P. Bornstein, University of Washington, Seattle) by selective salt precipitation.[15] Characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and densitometric scanning of the gels revealed less than 5% contamination with collagen type I. Collagen fibrils were prepared by dialysis against 20 mmol/L Na_2HPO_4.[16] Collagen concentration was determined by hydroxyproline analysis.[17] Optimal aggregation response in platelet-rich plasma was triggered with 2.5 μg/mL (final concentration).

Preparation of perfusates. Blood from normal donors was anticoagulated with ⅛ volume 108 mmol/L trisodium citrate. Platelets were washed, labeled with ^{51}Cr as previously described[18] and resuspended either in plasma (citrate concentration adjusted to 24 mmol/L) or in a human albumin salt solution containing 4% (wt/vol) albumin (No. A-9511, Sigma Chemicals Co, St Louis) in modified Tyrode buffer (130 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L $NaHCO_3$, pH 7.4) with 2.5 mmol/L $CaCl_2$, 0.9 mmol/L $MgCl_2$, and 5 mmol/L glucose. Traces of thrombin-contaminating albumin were removed by affinity chromatography on human anti-

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thrombin III (CRTS, Lille, France) coupled to Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) (1.5 mg antithrombin III per mL Sepharose 2B). Perfusates were reconstituted immediately prior to perfusion by adding washed red cells to the resuspended platelets (hematocrit 40%, platelet count 1.5×10^{11}/L).

Perfusion chamber, exposed surfaces, and perfusion procedures. A modification of the original parallel-plate perfusion chamber was used.[19] This chamber allows simultaneous exposure of the same perfusate to two collagen-coated cover slips positioned symmetrically on each side of the flow slit. Approximately 7 μg/cm^2 fibrillar type III collagen was sprayed onto plastic cover slips (Thermanox, Miles Laboratories Inc, Naperville, Ill) by means of an air brush (Model 100 II, Badger Air-brush Co, Franklin Park, Ill) at a nitrogen operating pressure of 1 atm.[19]

The collagen-coated surfaces were incubated 20 minutes at 22 °C with 0.4 mL of purified vWF, SpII and SpIII (15 μg/mL) dissolved in 25 mmol/L Tris-HCl, 150 mmol/L NaCl buffer, pH 7.4, containing 2.5 mmol/L $CaCl_2$ and 0.9 mmol/L $MgCl_2$.[20] Collagen-coated cover slips incubated with buffer served as control. The various surfaces were preperfused at 37 °C with 40 mL of modified Tyrode buffer containing 2.5 mmol/L $CaCl_2$, 0.9 mmol/L $MgCl_2$, and 5 mmol/L glucose, followed by perfusion with reconstituted blood perfusates at a flow rate of 41 mL/min corresponding to 2,600 seconds^{-1} shear rate for 5 minutes.[21]

Quantitation of platelet-collagen interactions. After perfusion, both cover slips were cut parallel to the flow axis in two equal halves. One half was used for ^{51}Cr counting, and platelet deposition was expressed as number of deposited platelets per cm^2.[21] The other half served for morphometric quantitation of platelet-collagen interactions. The percentage surface covered with platelets and microthrombi higher than 2.5 μm was estimated on semithin epon sections at 2 ± 1 mm downstream positions.[22] Fixation, embedding, and removal of exposed surfaces from cover slips for production of semithin sections were recently described in detail.[21]

Statistical analysis. Significance of grouped data was calculated with Student t-test, and P-values <0.05 were considered as significant.

RESULTS

Perfusion experiments using blood reconstituted with autologous citrated plasma. Preincubation of collagen with SpIII increased platelet deposition as effectively as purified vWF (Table 1). The number of deposited labeled platelets with SpIII and vWF compared with buffer showed an enhanced platelet deposition of about 4.5-fold ($P < 0.025$). Morphometric evaluation of the percentage surface coverage with adherent platelets paralleled the data obtained by ^{51}Cr counting (Table 1). The increased thrombus formation observed by preincubation of collagen with SpIII or vWF was not significant (Table 1).

Table 1. Effect of von Willebrand Factor Fragments Upon Platelet-Collagen Interactions in a Flow System (Shear Rate:2,600 seconds^{-1}) Using Blood Reconstituted With Citrated Plasma

Pre-incubation*	Number of Deposited Platelets (× 10^5/cm^2)†	% Surface Coverage: With Platelets†	% Surface Coverage: With Thrombi >2.5 μm†
Tris-HCl	26.8 ± 5.6	26.7 ± 5.3	4.2 ± 1.4
SpII	21.3 ± 2.4 (NS)	22.4 ± 2.6 (NS)	3.2 ± 1.0 (NS)
SpIII	122.3 ± 30.1‡	52.9 ± 8.6§	20.7 ± 6.3 (NS)
vWF	121.8 ± 27.8‡	78.0 ± 11.8‡	13.5 ± 6.0 (NS)

*Type III collagen (7 μg/cm^2) was preincubated at 20 °C for 20 minutes with purified proteins (15 μg/mL) or Tris-HCl buffer.

†Perfusions were performed with human blood reconstituted with citrated autologous plasma from three individuals and results represent mean ± SEM of three determinations in duplicate.

‡P-values compared with Tris-HCl buffer: $P < 0.025$.

§P-values compared with Tris-HCl buffer: $P < 0.05$.

Preincubation of the collagen-coated surface with SpII resulted in platelet-collagen interactions similar to those observed with buffer (Table 1).

Perfusion experiments using blood reconstituted with a human albumin salt solution. When citrated plasma was replaced by a purified albumin salt solution, preincubation of collagen-coated cover slips with SpIII again increased the platelet-collagen interactions. Similar results were obtained when using either SpIII or undigested vWF (Table 2). The percent surface coverage with platelets also paralleled the results obtained by ^{51}Cr counting (Table 2).

As in citrated plasma, preincubation of collagen-coated surfaces with SpII did not influence platelet-collagen interactions (Table 2).

DISCUSSION

vWF mediates platelet adhesion to subendothelium and to purified collagen at high wall shear rates that prevail in small vessels.[1] However, the respective role of plasma, platelet, and subendothelium vWF remains to be elucidated. Furthermore, the domains of vWF, a large multimeric protein with a complex structure that mediates platelet adhesion, are still unknown. We have shown in this study that a well characterized proteolytic fragment of vWF subunit, SpIII, mediates platelet adhesion to collagen at high shear rate whereas the complementary fragment SpII has no effect. The efficacy of SpIII in promoting platelet adhesion to fibrillar human collagen was established using both ^{51}Cr-platelets and a morphometric method to evaluate platelet-collagen interactions. In our experimental conditions using blood reconstituted with citrated plasma and vWF or purified SpIII coated on collagen, plasmatic vWF present in the perfusate did not appear to compete with bound material.

Table 2. Effect of von Willebrand Factor Fragments Upon Platelet-Collagen Interactions in a Flow System (Shear Rate:2,600 seconds^{-1}) Using Blood Reconstituted With a Human Albumin Salt Solution

Pre-incubation*	Number of Deposited Platelets (× 10^5/cm^2)†	% Surface Coverage: With Platelets†	% Surface Coverage: With Thrombi >2.5 μm†
Tris-HCl	58.6 ± 8.6	33.7 ± 5.4	5.2 ± 2.5
SpII	62.7 ± 11.1 (NS)	21.8 ± 4.2 (NS)	6.3 ± 2.0 (NS)
SpIII	159.4 ± 12.0‡	92.9 ± 1.4‡	20.1 ± 10.3 (NS)
vWF	153.4 ± 8.1‡	95.0 ± 1.2§	17.4 ± 10.3 (NS)

*Type III collagen (7 μg/cm^2) was preincubated at 20 °C for 20 minutes with purified proteins (15 μg/mL) or Tris-HCl buffer.

†Perfusions were performed with human blood from three individuals reconstituted with 4% albumin solution in modified Tyrode buffer containing physiologic amounts of divalent cations. Results represent mean ± SEM of three determinations in duplicate.

‡P-values compared with Tris-HCl buffer: $P < 0.0025$.

§P-values compared with Tris-HCl buffer: $P < 0.0005$.

SpIII contains a binding domain for platelet membrane GPIb.[10] Our study suggests that it also possesses a domain for binding to collagen, confirming results of other studies obtained with monoclonal antibodies (MoAb) to vWF. We showed that among 24 MoAb to vWF, six of them (B200 through B205) specifically inhibit binding of vWF to collagen[23] while two other MoAb, MoAb H9 and MoAb 9, inhibit vWF binding to ristocetin- and to thrombin-stimulated platelets, respectively.[24,25] Thus SpIII, already known to contain a binding domain for GPIb, also possesses a distinct site for binding to collagen, and both domains appear to be required for platelet-collagen interactions at high shear rate. Contrary to SpIII, SpII which binds to GPIIb/IIIa does not appear to be involved, suggesting that it contains no collagen-binding domain or no platelet-binding domain implicated in platelet adhesion. Alternatively, the GPIIb/IIIa binding sites for SpII may not be induced on the platelet membrane in our experimental conditions; at high shear rate, binding to GPIb may be a prerequisite for platelet activation leading to exposure of GPIIb/IIIa.

The finding that SpIII supports platelet attachment to collagen is further substantiated by perfusion experiments using blood reconstituted with a salt solution containing albumin as the only exogenous protein. The enhanced effect of SpIII or vWF as compared with experiments with blood reconstituted with autologous citrated plasma may be related to the presence of physiologic amounts of calcium added to the human albumin solution. Indeed, the importance of divalent cations in platelet interaction with subendothelium has been emphasized.[18] Our results confirm previous findings[20] that in vitro, at high shear rate, vWF appears to be the only protein required for mediating platelet interactions with fibrillar collagen and stress the relative importance of locally adsorbed vWF as compared with circulating vWF.

In conclusion, the *N*-terminal fragment SpIII of vWF mediates platelet adhesion to collagen as well as undigested vWF. The multimeric structure of vWF thus does not appear to be required for platelet adhesion to fibrillar collagen in vitro. The possibility, however, that SpIII aggregates on the collagen to provide a repetitive structure has not been excluded. Finally, our results do not stipulate that in vivo the large vWF multimers are unimportant for normal hemostasis but only indicate that in vitro a specific fragment may substitute for the fully polymerized vWF in promoting platelet adhesion to collagen.

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REFERENCES

1. Meyer D, Baumgartner HR: Role of von Willebrand factor in platelet adhesion to the subendothelium. Br J Haematol 54:1, 1983

2. Kao KJ, Pizzo SV, McKee PA: Demonstration and characterization of specific binding sites for factor VIII/von Willebrand factor on human platelets. J Clin Invest 63:656, 1979

3. Fujimoto T, Ohara S, Hawiger J: Thrombin-induced exposure and prostacyclin inhibition of the receptor for factor VIII/von Willebrand factor on human platelets. J Clin Invest 69:1212, 1982

4. Weiss HJ, Tschopp TB, Baumgartner HR, Sussman II, Johnson MM, Egan JJ: Decreased adhesion of giant (Bernard-Soulier) platelets to subendothelium: Further implications on the role of the von Willebrand factor in hemostasis. Am J Med 57:920, 1974

5. Tschopp TB, Weiss HJ, Baumgartner HR: Decreased adhesion of platelets to subendothelium in von Willebrand's disease. J Lab Clin Med 83:296, 1974

6. Stel HV, Sakariassen KS, de Groot PG, van Mourik JA, Sixma JJ: von Willebrand factor in the vessel wall mediates platelet adherence. Blood 65:85, 1985

7. Bolhuis PA, Sakariassen KS, Sander HJ, Bouma BN, Sixma JJ: Binding of factor VIII-von Willebrand factor to human arterial subendothelium precedes increased platelet adhesion and enhances platelet spreading. J Lab Clin Med 97:568, 1981

8. Sixma JJ, Sakariassen KS, Stel HV, Houdjik WPM, in der Maur DW, Hamer RJ, de Groot PG, van Mourik J: Functional domains on von Willebrand factor. Recognition of discrete tryptic fragments by monoclonal antibodies that inhibit interaction of von Willebrand factor with platelets and with collagen. J Clin Invest 74:736, 1984

9. Girma JP, Pietu G, Chopek MW, Edgington TS, Meyer D: Localization of distinct Willebrand factor domains interacting with ristocetin or thrombin stimulated platelets using proteolysis and monoclonal antibodies. Circulation 70:836A, 1984 (abstr)

10. Girma JP, Kalafatis M, Pietu G, Lavergne JM, Chopek MW, Edgington TS, Meyer D: Mapping of distinct von Willebrand factor domains interacting with platelet GPIb and GPIIb/IIIa and with collagen using monoclonal antibodies. Blood (in press)

11. Girma JP, Chopek MW, Titani K, Davie EW: Limited proteolysis of human von Willebrand factor by S. aureus V-8 protease: Isolation and partial characterization of a platelet-binding domain. Biochemistry (in press)

12. Meyer D, Obert B, Pietu G, Lavergne JM, Zimmerman TS: Multimeric structure of Factor VIII/von Willebrand factor in von Willebrand's disease. J Lab Clin Med 95:590, 1980

13. Chopek MW, Girma JP, Fujikawa K, Davie EW, Titani K: Human von Willebrand factor: A multivalent protein composed of identical subunits. Biochemistry (submitted)

14. Ardaillou N, Girma JP, Meyer D, Lavergne JM, Shoa'i I, Larrieu MJ: "Variants" of von Willebrand disease. Demonstration of a decreased antigenic reactivity by immunoradiometric assay. Thromb Res 12:817, 1978

15. Miller EF, Rhodes RK: Preparation and characterization of the different types of collagen. Methods Enzymol 82:33, 1982

16. Bruns RR, Gross J: High-resolution analysis of the modified quarter-stagger model of the collagen fibril. Biopolymers 13:931, 1974

17. Woessner JF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 93:440, 1961

18. Sakariassen KS, Ottenhof-Rovers M, Sixma JJ: Factor VIII-von Willebrand factor requires calcium for facilitation of platelet adherence. Blood 63:996, 1984

19. Sakariassen KS, Aarts PA, de Groot PG, Houdijk WP, Sixma JJ: A perfusion chamber developed to investigate platelet interaction in flowing blood with human vessel wall cells, their extracellular matrix and purified components. J Lab Clin Med 102:522, 1983

20. Houdijk WP, Sakariassen KS, Nievelstein PF, Sixma JJ: Role of factor VIII-von Willebrand factor and fibronectin in the interaction of platelets in flowing blood with monomeric and fibrillar human collagen types I and III. J Clin Invest 75:531, 1985

21. Sakariassen KS, Muggli R, Baumgartner HR: Measurements of platelet interaction with components of the vessel wall in flowing blood. Methods Enzymol (in press)

22. Baumgartner HR, Muggli R: Adhesion and aggregation: Morphological demonstration and quantitation in vivo and in vitro, in Gordon JL (ed): Platelets in Biology and Pathology. Amsterdam, Elsevier, North-Holland, 1976, p 23

23. Pietu G, Fressinaud E, Girma JP, Nieuwenhuis HK, Rothschild C, Meyer D: Binding of human von Willebrand factor to collagen and to collagen stimulated platelets. J Lab Clin Med (submitted)

24. Meyer D, Baumgartner HR, Edgington TS: Hybridoma antibodies to human von Willebrand factor. II. Relative role of intramolecular loci in mediation of platelet adhesion to the subendothelium. Br J Haematol 57:609, 1984

25. Nokes TJC, Mahmoud NA, Savidge GF, Goodall AH, Meyer D, Edgington TS, Hardisty RM: von Willebrand factor has more than one binding site for platelets. Thromb Res 34:361, 1984

CONCISE REPORT

Invasion of Erythrocytes by *Plasmodium falciparum* Malaria Parasites: Evidence for Receptor Heterogeneity and Two Receptors

By Graham H. Mitchell, Terence J. Hadley, Mary H. McGinniss, Francis W. Klotz, and Louis H. Miller

Plasmodium falciparum malaria parasites with different capabilities of invading sialic acid-deficient erythrocytes were identified. Thai-2 parasites cultured in Tn erythrocytes invaded neuraminidase-treated and Tn erythrocytes twice as efficiently as Thai-2 parasites cultured in normal erythrocytes and seven to ten times more efficiently than a cloned line of Camp parasites cultured in normal erythrocytes. All three parasite lines required sialic acid for optimal invasion, but Thai-2 parasites cultured in Tn erythrocytes invaded neuraminidase-treated erythrocytes with 45% efficiency whereas Camp parasites invaded neuraminidase-treated erythrocytes with less than 10% efficiency. *P falciparum* malaria parasites probably possess two receptors: one that binds to a sialic acid-dependent ligand and another that binds to a sialic acid-independent ligand. Parasites may differ in the quantity or affinity of their receptors for the sialic acid-independent ligand.

MALARIA is caused by protozoa (*Plasmodium*) that parasitize erythrocytes. *Plasmodium falciparum* is especially important as a cause of morbidity and mortality in tropical areas of the world. Invasion of erythrocytes by malaria parasites involves specific interactions between parasite receptors and erythrocyte ligands.[1] Sialic acid on the erythrocyte membrane is important for optimal invasion by *P falciparum* parasites. Neuraminidase-treated erythrocytes and Tn erythrocytes, which lack sialic acid and galactose on the O-linked tetrasaccharides of the glycophorins,[2-4] are less susceptible to invasion than normal erythrocytes.[5-9] The present study was done to determine whether the requirement for erythrocyte sialic acid varies among strains of *P falciparum* and whether erythrocyte ligands other than sialic acid are important for invasion.

P falciparum parasites from an uncloned isolate were cultured in Tn erythrocytes and examined for their ability to invade neuraminidase-treated erythrocytes, Tn erythrocytes, and trypsin-treated erythrocytes. Comparisons were made with the parent line cultured in normal erythrocytes and with a cloned line from another strain. The observations reported here indicate that *P falciparum* parasites vary in their abilities to invade sialic acid-deficient erythrocytes and that in addition to sialic acid, another erythrocyte ligand is required for invasion.

MATERIALS AND METHODS

Cultures and parasites. Tn erythrocytes from donor C.L. were provided by Mary L. Thomas, United Blood Services, Billings, Mont. *P falciparum* parasites were cultured in vitro as previously described.[10,11] Human serum used in the culture medium was preabsorbed overnight at 4 °C with 4% vol/vol C.L. erythrocytes in order to remove naturally occurring anti-Tn antibodies. An uncloned isolate (Thai-2) of *P falciparum* from Thailand was cultured in C.L. erythrocytes and designated Thai-Tn. Separate cultures of the Thai-2 line were maintained in normal erythrocytes. Cultures of the Malayan-Camp strain were also maintained in normal erythrocytes. This line was derived from a cloned parasite (J.D. Haynes, MD, unpublished data, April, 1982) and did not survive when cultured in Tn erythrocytes.

Quantitation and purification of Tn erythrocytes. Erythrocytes from donor C.L. were agglutinated by *Salvia sclarea* lectin, which binds to N-acetylgalactosamine residues exposed on Tn erythrocytes.[12] About 10% of C.L. erythrocytes contained the M blood group antigen as determined by immunofluorescence with an anti-M monoclonal antibody, indicating that approximately 90% of C.L. erythrocytes were of the Tn phenotype.[4] To remove normal erythrocytes, C.L. blood was incubated with the anti-M monoclonal antibody (a gift of J.J. Moulds, Gamma Biologicals, Houston, Tex) and passed over an immunoaffinity column containing goat antimouse immunoglobulin covalently coupled to Sepharose 4B (Cappel, Cochranville, Penn). Fewer than 1% of the effluent cells possessed M antigens as determined by immunofluorescence.

Treatment of erythrocytes with enzymes. Erythrocytes (1×10^8/mL) were treated with various concentrations of neuraminidase (*Vibrio cholerae.*, GIBCO, Grand Island, NY) in RPMI 1640, 23 mmol/L Hepes, pH 7.2, for one hour at 37 °C. Concentrations from 5 to 100 U/mL were equally effective at reducing invasion of erythrocytes by the malarial parasites tested. Results of the first experiment are expressed as averages of the results obtained at each of the concentrations of neuraminidase (5, 10, 25, 75, and 100 U/mL). In all subsequent experiments, neuraminidase was used at a concentration of 50 U/mL. Erythrocytes (1×10^8/mL) were treated with trypsin-TPCK (L-1-tosylamide-2-phenylethyl-chloromethyl ketone; type XIII, Sigma; 1 mg/mL) at 37 °C for one hour; trypsin-treated erythrocytes were washed in RPMI 1640, 23 mmol/L Hepes, and the remaining trypsin was inhibited with soybean trypsin inhibitor (Sigma; 1 mg/mL) prior to final washes.

Invasion Assay. Malaria parasites (merozoites) invade erythrocytes and develop sequentially into ring-forms, trophozoites, and schizonts. Each schizont in culture produces approximately ten to 20 new merozoites, which are released when infected erythrocytes rupture and invade other erythrocytes. Schizont-infected erythrocytes were obtained[13] and purified (99% purity) on Percoll-sorbitol

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gradients according to the method of Aley.[14] Invasion assays were done in flat-bottom microtiter wells containing 175 μL culture medium, 1×10^6 schizont-infected erythrocytes, and 1×10^7 uninfected erythrocytes. Sera used in the culture media were absorbed with Tn and neuraminidase-treated erythrocytes to remove anti-Tn and anti-T antibodies, respectively. Cultures were harvested after 18 hours for the preparation of blood films, which were fixed with methanol, stained with Giemsa, and examined by light microscopy. For each culture, the percent of erythrocytes infected with ring-forms (invasion rate) was determined by examining 1,000 erythrocytes. Efficiency of invasion was defined as the invasion rate obtained with enzyme-treated or variant erythrocytes expressed as a percentage of the invasion rate obtained with normal erythrocytes using the same line of parasite.

RESULTS

In preliminary experiments four different lines of *P. falciparum*, including the cloned Camp line, were cultured in erythrocytes from donor C.L. (90% Tn). Only the Thai-2 line survived and multiplied. Thai-2 parasites maintained in culture with Tn erythrocytes (Thai-Tn) were compared with Thai-2 parasites maintained in normal erythrocytes and with Camp parasites maintained in normal erythrocytes. Thai-Tn parasites invaded neuraminidase-treated erythrocytes with an average efficiency of 45%, in contrast to Camp parasites, which invaded neuraminidase-treated erythrocytes with an average efficiency of about 5% (Table 1). Thai-2 parasites invaded neuraminidase-treated erythrocytes with an average efficiency of 28% (Table 1). All three lines tested required sialic acid for optimal invasion. Similar differences were obtained with Tn erythrocytes (Table 2). Thai-Tn parasites invaded Tn erythrocytes seven to eight times more efficiently than did Camp parasites and two to three times more efficiently than did Thai-2 parasites. Furthermore, treatment of Tn erythrocytes with neuraminidase increased their susceptibility to invasion by Thai-Tn parasites by about 40% but had no effect on the susceptibility of these erythrocytes to invasion by Camp parasites. Treatment of normal erythrocytes with trypsin decreased susceptibility to invasion by all three lines tested to about 10% of normal (Table 1).

Table 1. Invasion of Enzyme-Treated Normal Erythrocytes by Various Lines of *Plasmodium falciparum*

Treatment	Experiment No.	Invasion Expressed* as % of Control: Camp	Thai-2	Thai-Tn	Ratio† of Thai-Tn:Camp
Neuraminidase	1	1		25	25
	2		13	47	
	3	7	33	54	7.7
	4	6	28	53	8.8
Trypsin	1	12		10	0.8
	2		15	4	

*Invasion rates obtained with test RBCs are expressed as a percentage of invasion rates obtained with normal, untreated RBCs and are calculated as follows:

$$\frac{\text{\% of test RBCs infected}}{\text{\% of normal RBCs infected}} \times 100$$

Invasion rates obtained with normal RBCs were greater than 10% in most experiments. The data are presented for three lines of parasites: Camp, Thai-2, and Thai-Tn (Thai-2 parasites cultured in Tn RBCs).

†The ratios of the invasion rates of Thai-Tn parasites to Camp parasites in the same population of RBCs.

Table 2. Invasion of Purified Tn Erythrocytes and Purified Tn Erythrocytes Treated with Neuraminidase by Various Lines of *Plasmodium falciparum*

Treatment	Experiment No.	Invasion Expressed* as % of Control: Camp	Thai-2	Thai-Tn	Ratio† of Thai-Tn:Camp
None	3	2	5	15	7.5
	4	2	8	19	9.5
	5	3		20	6.7
Neuraminidase	4	2	15	31	15.5
	5	1		29	29

*†Same as Table 1.

DISCUSSION

Thai-Tn parasites invaded neuraminidase-treated erythrocytes with 45% efficiency and Thai-2 parasites invaded neuraminidase-treated erythrocytes with 28% efficiency Although both parasites required sialic acid on the erythrocyte membrane for optimal invasion, their ability to invade neuraminidase-treated erythrocytes suggest that they can also utilize an erythrocyte ligand which is independent of sialic acid. The low efficiency of invasion of neuraminidase-treated erythrocytes obtained with Camp parasites may be due to residual sialic acid on the erythrocyte membrane after neuraminidase treatment, or to a low affinity interaction with another ligand. Treatment of Tn erythrocytes with neuraminidase enhanced their susceptibility to invasion by Thai-Tn parasites but not by Camp parasites, further indicating that Thai-Tn and Camp parasites have different receptors and that Thai-Tn parasites can utilize a sialic acid-independent ligand. The increased invasion of Tn erythrocytes by Thai-Tn parasites following neuraminidase treatment may be due to the decrease in negatively charged residues (sialic acid), making a second erythrocyte ligand more accessible.

The data presented here is the first reported indication that there is receptor heterogeneity among *P falciparum* parasites. Previous reports suggested that two receptors may be involved in invasion.[7,15,16] Friedman et al reported that the effect of neuraminidase on invasion could be partially reversed by incubating neuraminidase-treated erythrocytes with α_1 acid glycoprotein.[7] This effect, which required sialic acid on α_1 acid glycoprotein, did not occur when α_1 acid glycoprotein was incubated with trypsin-treated erythrocytes. Other investigators suggested that, in addition to sialic acid, the hydrophobic T_6 peptide of glycophorin A may be a ligand necessary for invasion because the T_6 peptide inhibits invasion in vitro.[15,16] However, the specificity of inhibition by the hydrophobic T_6 peptide has been questioned by Breuer et al, who found that hydrophobic haptens coupled to bovine serum albumin also inhibit invasion.[17] The T_6 peptide of glycophorin A remains associated with the membrane after trypsin treatment of intact erythrocytes and therefore is probably not the trypsin-sensitive ligand utilized by Thai-Tn, Thai 2, and possibly Camp parasites.

The data presented here suggest that *P falciparum* malaria parasites express at least two different receptors for two different ligands on the erythrocyte membrane. One receptor binds to a ligand that is dependent on sialic acid, and a second receptor binds to a ligand that is independent of sialic acid and is sensitive to cleavage by trypsin. Our observations suggest that Thai-Tn parasites have either a greater number of receptors for the sialic acid-independent ligand than Camp parasites or receptors of higher affinity. The location of the sialic acid-independent ligand is unknown. The biologic evidence for two receptors and for receptor heterogeneity may be important to future biochemical characterization of receptors and to vaccine development if receptors are to be used as immunogens.

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REFERENCES

1. Miller LH, Haynes JD, McAuliffe FM, Shiroishi T, Durocher JR, McGinniss MH: Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. J Exp Med 146:277, 1977

2. Dahr W, Uhlenbruck G, Gunson HH, van der Hart M: Molecular basis of Tn-polyagglutinability. Vox Sang 29:36, 1975

3. Cartron JP, Andrew G, Cartron J, Bird GWG, Salmon C, Gerbal A: Demonstration of T-transferase deficiency in Tn-polyagglutinable blood samples. Eur J Biochem 92:111, 1978

4. Anstee DJ: The blood group MNSs-active sialoglycoproteins. Semin Hematol 18:13, 1981

5. Perkins ME: Inhibitory effects of erythrocyte membrane proteins on the *in vitro* invasion of the human malarial parasite (*Plasmodium falciparum*) into its host cell. J Cell Biol 90:563, 1981

6. Breuer WV, Ginsburg H, Cabantchik ZI: An assay of malaria parasite invasion into human erythrocytes. The effects of chemical and enzymatic modification of erythrocyte membrane components. Biochim Biophys Acta 755:263, 1983

7. Friedman MJ, Blankenberg T, Sensabaugh G, Tenforde TS: Recognition and invasion of human erythrocytes by malarial parasites: Contribution of sialoglycoproteins to attachment and host specificity. J Cell Biol 98:1672, 1984

8. Pasvol G, Jungery M, Weatherall DJ, Parsons SF, Anstee DJ, Tanner MJA: Glycophorin as a possible receptor for *Plasmodium falciparum*. Lancet ii 947:950, 1982

9. Cartron JP, Prou O, Luilier M, Soulier JP: Susceptibility to invasion by *Plasmodium falciparum* of some human erythrocytes carrying rare blood group antigens. Br J Haematol 55:639, 1983

10. Haynes JD, Diggs CL, Hines FA, Desjardins RE: Culture of human malaria parasites *Plasmodium falciparum*. Nature 263:767, 1976

11. Chulay JD, Haynes JD, Diggs CL: Serotypes of *Plasmodium falciparum* defined by immune serum inhibition of *in vitro* growth. Bull WHO 63:317, 1985

12. Bird GWG, Wingham J: Hemagglutinins from *Salvia*. Vox Sang 26:163, 1974

13. Lambros C, Vanderberg JP: Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol 65:418, 1979

14. Aley SB, Sherwood JA, Howard RJ: Knob-positive and knob-negative *Plasmodium falciparum* differ in expression of a strain-specific malarial antigen on the surface of infected erythrocytes. J Exp Med 160:1585, 1984

15. Breuer WV, Kahane I, Baruch D, Ginsburg H, Cabantchik ZI: Role of internal domains of glycophorin in *Plasmodium falciparum* invasion of human erythrocytes. Infect Immun 42:133, 1983

16. Perkins ME: Binding of glycophorins to *Plasmodium falciparum* merozoites. Mol Biochem Parasitol 10:67, 1984

17. Breuer WV, Ginsburg H, Cabantchik ZI: Hydrophobic interactions in *Plasmodium falciparum* invasion into human erythrocytes. Mol Biochem Parasitol 12:125, 1984

CORRESPONDENCE

TRANSCOBALAMIN II DEFICIENCY AND ORAL COBALAMIN THERAPY

To the Editor:

The interesting report by Zeitlin and associates[1] of a new case of transcobalamin II (TC-II) deficiency raises several issues worth considering further. The most striking of their observations is the apparent symptom-free maintenance of their patient with oral cobalamin therapy. A note of caution is necessary, however. While various regimens may keep TC-II-deficient patients symptom-free and overtly normal, using the minimal therapy that does so may not be ideal, particularly in the developmental years. Less clinically apparent organ dysfunction may remain incompletely corrected. An obvious example may be persistent cobalamin malabsorption whose mechanism is unknown but could be due to cobalamin depletion of gut cells, as suggested previously.[2] Malabsorption of other substances may conceivably also exist. Another possibility of uncorrected long-term dysfunction could be in the reproductive system. Since TC-II is present in semen in greater concentrations than everywhere else[3] (its presence in the female reproductive system has not been studied but may be similar), reproductive function may be particularly sensitive to TC-II and cobalamin depletion. The answers to such concerns are not yet available. Subtle dysfunction of various organ systems may not become evident until more time has elapsed. While oral therapy has obvious advantages over injections, our limited means of assessing cobalamin status in most tissues dictates caution in deciding on ideal therapy.

The albumin-sized binder of cobalamin that Zeitlin et al[1] observed is also noteworthy. In the two cases of TC-II deficiency that my associates and I recently described in considerable detail,[4,5] relatively large amounts of this binder, in both "apo" and "holo" form, were found before treatment. We found that this binding seemed to increase greatly with cobalamin therapy. What makes the albumin-sized binder even more intriguing is that slightly increased amounts also seemed to occur in the heterozygous relatives of our TC-II-deficient patients.[4,5] R-binder deficient patients also have detectable albumin-sized binder[6] as may other patients occasionally,[7] but none to the same extent that homozygous TC-II-deficient patients do, particularly after therapy. It remains unclear whether such elevation is a compensatory mechanism, a part of the binder abnormality syndrome, or merely the unmasking of a relatively common phenomenon.

Finally, I would like to comment on a technical point and on the unclearness of whether the patient described by Zeitlin et al had total absence of TC-II or not. It is unfortunate that fluoride-EDTA anticoagulant was used. Since this anticoagulant causes osmotic dehydration of red cells and significant dilution of plasma, plasma values are regularly falsely lowered.[8] The need to anticoagulate the blood sample arises only when the R-binders are of concern. An ideal anticoagulant for that purpose has not been found, but EDTA alone may be the best available choice. When TC-II is being studied, serum serves as well as plasma. Although Zeitlin et al[1] state that their patient had total absence of TC-II, their Table 1 indicates that she had some TC-II, 6.5 to 21 ng/L of unsaturated TC-II by chromatographic determination and ~50 ng/L of total TC-II by radioimmunoassay; moreover, these amounts were probably underestimated because of the choice of anticoagulant. If indeed present, even in such greatly reduced amounts, this TC-II might explain the patient's responsiveness to oral cobalamin therapy.

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REFERENCES

1. Zeitlin HC, Sheppard K, Baum JD, Bolton FG, Hall CA: Homozygous transcobalamin II deficiency maintained on oral hydroxocobalamin. Blood 66:1022, 1985

2. Carmel R: Megaloblastic anemia: Vitamin B_{12} and folate, in Fairbanks V (ed): Current Hematology, vol 2. New York, Wiley, 1983, p 243

3. Carmel R, Bernstein GS: Transcobalamin II in human seminal plasma. J Clin Invest 73:868, 1984

4. Carmel R, Ravindranath Y: Congenital transcobalamin II deficiency presenting atypically with a low serum cobalamin level: Studies demonstrating the coexistence of a circulating transcobalamin I (R binder) complex. Blood 63:598, 1984

5. Meyers PA, Carmel R: Hereditary transcobalamin II deficiency with subnormal serum cobalamin levels. Pediatrics 74:866, 1984

6. Carmel R: A new case of deficiency of the R binder for cobalamin, with observations on minor cobalamin-binding proteins in serum and saliva. Blood 59:152, 1982

7. Carmel R: The distribution of endogenous cobalamin among cobalamin-binding proteins in the blood in normal and abnormal states. Am J Clin Nutr 41:713, 1985

8. Carmel R: Vitamin B_{12}-binding proteins in serum and plasma in various disorders. Effects of anticoagulants. Am J Clin Pathol 69:319, 1978

To the Editor:

In reply to Dr Carmel's letter, the best evidence we have for a total absence of transcobalamin II (TC-II) in our patient comes from radioimmunoassay. The value obtained was below the first point on the calibration graph and addition of further serum from the patient made no difference. We have no immunologic confirmation that the small amount of protein binding cobalamin in the TC-II position on chromatographic determination is in fact TC-II. We agree that fluoride-EDTA is not the best anticoagulant for TC-II estimations; however, there was no significant difference in the TC-II unsaturated B_{12} binding capacity when we used serum instead of fluoride-EDTA plasma.

That tissue requirement for cobalamin appears variable is well illustrated by our case and we take the point that the present dose of hydroxocobalamin (1 mg twice daily) may not be sufficient to ensure normal reproduction or indeed growth in adolescence. However, there is no reason that this note of caution should have any bearing on the route of administration of cobalamin. Our patient is taking oral hydroxocobalamin in comparable dosage to other patients already described who are being treated parenterally, and we would stress again that 12.5 μg cyancobalamin daily by mouth produced blood cobalamin levels of >1,000 μg/L and that 1 mg twice daily of hydroxocobalamin produced blood levels of 3,000 to 4,000 μg/L.

From our experience with this case and the previous literature, we believe that the total dose and the frequency of cobalamin administration are the most important factors in maintaining adequate cobalamin status in TC-II deficiency.

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VON WILLEBRAND FACTOR IN THROMBOTIC THROMBOCYTOPENIC PURPURA

To the Editor:

A report by Lian and Siddiqui in the Nov 1985 issue of *Blood*[1] claimed to provide evidence that von Willebrand factor (vWF) is not involved in the pathophysiology of acute thrombotic thrombocytopenic purpura (TTP) episodes. The experiments described in the article did not justify the authors' conclusion. Lian and Siddiqui found a platelet clumping activity in the plasma obtained from five patients during TTP episodes. They reported that this clumping activity was not neutralized by preincubation of acute TTP patient plasma with either polyclonal antibody to vWF or by incubation of test platelets with monoclonal antibody to glycoprotein Ib. (Glycoprotein Ib is one of several externally exposed platelet membrane proteins to which vWF multimers will bind, or bind near, on the platelet surface. Others are the glycoprotein IIb-IIIa complex and a separate protein of mol wt 210,000.)[2,3]

When large vWF multimers attach to platelets in vivo during acute TTP episodes, large vWF forms decrease or disappear from patient plasma. This has been found in samples obtained during TTP episodes in eight studies from four different laboratories.[4-11] In the paper by Lian and Siddiqui,[1] vWF multimeric forms in patient plasma were not reported. It is likely that large vWF multimeric forms had already decreased or disappeared from their patient samples before they were obtained. Interference with agglutination by antibodies against vWF would not, therefore, have been expected under their experimental conditions, ie, the mixing of washed normal platelets, anti-vWF antibodies, and acute TTP plasma samples devoid of the large forms of vWF that bind to platelets.

Because of the probable relative absence of large vWF multimeric forms in the acute TTP plasma samples of Lian and Siddiqui's patients, I would also have expected that an antibody against one of the platelet glycoprotein receptors for large vWF multimers would have no effect on platelet clumping. By the same reasoning, antibody against the glycoprotein IIb-IIIa complex would have been expected to have no effect under their particular test conditions. Nevertheless, it would have been important to determine if antibody directed against one of the other potential binding sites for large vWF multimers had any effect in their test system.

Lian and Siddiqui's experiments were based entirely on the in vitro clumping of platelets in the presence of several plasma samples obtained during acute TTP episodes. Platelet clumping in their test system was, apparently, relatively modest in the presence of acute TTP plasma alone. The actual extent of aggregation is not given in their article.[1] However, 20% to 32% change in light transmission was reported in one of their previous publications (Table 1 of reference 12). Platelet aggregation of this limited extent is difficult to reproduce. This is especially true if (as in their system) the test platelets have been separated, washed, and resuspended in buffer before being suspended in TTP plasma.

The factor that causes the slight amount of platelet clumping in the plasma samples of Lian and Siddiqui is not clear. The authors present no direct evidence to indicate that it is not an isoantibody. It has long been known that plasma samples will clump normal donor platelets if the plasma contains isoantibodies directed against histocompatibility antigens on the surface of test platelets.[13,14] Platelet clumping tests performed on TTP plasma samples are probably useful only as relatively insensitive screening tests for the presence of platelet isoantibodies when the aggregometry studies are performed using plasma from patients who have previously been transfused with blood products, or who have previously been pregnant. Lian and Siddiqui[1] do not indicate if their five TTP patients had ever been transfused or pregnant prior to the acquisition of their plasma samples for testing. They also do not mention if they have tested for the presence of HLA isoantibodies in the TTP patient plasma samples. Other investigators have observed that in vitro platelet clumping in some TTP plasma samples is probably caused by immunoglobulins.[15] It is possible, therefore, that the platelet clumping test used by Lian and Siddiqui,[1] which employs plasma from TTP patients and platelets from normal (non-HLA identical) donors, predominantly detects platelet isoantibodies in TTP patient plasma samples capable of clumping non-HLA-identical normal platelets in vitro. The authors do not indicate that they controlled for this possibility by incubating their TTP plasma samples with antihuman IgG, IgM, and IgA before performing platelet clumping studies.

Along with others,[16] I am skeptical that the in vitro platelet clumping in the test of Lian and Siddiqui is related to the pathophysiology of TTP episodes in vivo. Platelet clumping observed in their test system, whether induced by acute TTP plasma or by fractions purified from it, is claimed by Lian et al to be inhibited by normal human IgG.[12] Lian and colleagues[17] have suggested, from the clinical course of one patient, that intravenous (IV) immunoglobulin may be useful in vivo in the treatment of acute episodes of TTP. However, Miuri et al[6] as well as Dr J.J. Byrnes (Miami) and Dr J.G. Kelton (Hamilton, Ontario) in unpublished studies have found that concentrated IV IgG is of no therapeutic value in vivo during acute TTP episodes, and that IV IgG does not truncate or delay relapses in the chronic relapsing form of the disorder.

My colleagues and I,[4,8-10] as well as Kelton et al[7,11] and others[5,6] have concluded that vWF is likely to be involved in the pathophysiology of TTP and closely related syndromes.[18] The largest plasma vWF multimeric forms have been found to be depleted in the plasma of TTP patients tested during the in vivo platelet clumping and thrombocytopenia that characterize acute TTP episodes.

Kelton et al[7] have also observed that acute TTP serum, which does not directly agglutinate normal donor washed platelets, can be made to do so by the addition of normal defibrinated cryoprecipitate (containing the largest plasma vWF multimeric forms). They found that neither the smallest plasma vWF forms nor fibronectin supports platelet agglutination in the presence of acute TTP serum. In contrast to the report by Lian et al,[12] platelet clumping in the test system of Kelton et al[7] is not inhibited by normal IgG.

Plasma from which the large vWF multimeric forms have been depleted (ie, cryosupernatant) is effective treatment for acute TTP episodes, is effective prophylaxis against recurrence in chronic relapsing TTP patients, and is often effective therapy for acute episodes of TTP when other types of plasma manipulation therapy have failed.[9,16,19] The therapeutic effectiveness of cryosupernatant, along with laboratory observations made by a number of investigators,[4-11] are compatible with the conclusion that large vWF forms are involved in the pathophysiology of TTP. It is not yet known if the attachment to platelets of large vWF multimeric forms is of itself sufficient to induce platelet agglutination in vivo, or if fibrinogen, fibronectin, or other plasma proteins (or protein fragments) are also involved.

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REFERENCES

1. Lian EC-Y, Siddiqui FA: Investigation of the role of von Willebrand factor in thrombotic thrombocytopenic purpura. Blood 66:1219, 1985

2. Lombardo VT, Hodson E, Roberts JR, Kunicki TJ, Zimmerman TS, Ruggeri ZM: Independent modulation of von Willebrand factor and fibrinogen binding to the platelet membrane glycoprotein IIb-IIIa complex as demonstrated by monoclonal antibody. J Clin Invest 76:1950, 1985

3. Stricker RB, Wong D, Saks SS, Corash L, Shuman MA: Acquired Bernard-Soulier syndrome. Evidence for the role of 210,000-molecular weight protein in the interaction of platelets with von Willebrand factor. J Clin Invest 76:1274, 1985

4. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, Seder RH, Hong SL, Deykin D: Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med 307:1432, 1982

5. Rowe JM, Francis CW, Cyran EM, Marder VJ: Thrombotic thrombocytopenic purpura: Recovery after splenectomy associated with persistence of abnormally large von Willebrand factor multimers. Am J Hematol 20:161, 1985

6. Miura M, Koizumi S, Nakamura K, Ohno T, Tachinami T, Yamagami M, Taniguchi N, Kinoshita S, Abildgaard CF: Efficacy of several plasma components in a young boy with chronic thrombocytopenia and hemolytic anemia who responds repeatedly to normal plasma infusions. Am J Hematol 17:307, 1984

7. Kelton JG, Moore J, Santos A, Sheridan D: The detection of a platelet-agglutination factor in thrombotic thrombocytopenic purpura. Ann Intern Med 101:589, 1984

8. Moake JL, Rudy CK, Troll JH, Schafer AI, Weinstein MJ, Colannino NM, Hong SL: Therapy of chronic relapsing thrombotic thrombocytopenic purpura with prednisone and azathioprine. Am J Hematol 20:73, 1985

9. Moake JL, Byrnes JJ, Troll JH, Rudy CK, Hong SL, Weinstein MJ, Colannino NM: Effects of fresh-frozen plasma and its cryosupernatant fraction on von Willebrand factor multimeric forms in chronic relapsing thrombotic thrombocytopenic purpura. Blood 65:1232, 1985

10. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Hong SL, Koutcher JA, Melaragno AJ, Manner CE: von Willebrand factor abnormalities and endothelial cell perturbation in acute thrombotic thrombocytopenic purpura. Am J Med Sci (in press)

11. Murphy WG, Moore JC, Barr RD, Pai MKR, Kelton JG: Relationship between the presence of a platelet aggregating factor and abnormalities of factor VIII: von Willebrand's factor in a patient with chronic thrombotic thrombocytopenic purpura. Blood 66:294, 1985 (suppl 1) (abstr)

12. Lian EC, Mui PT, Siddiqui FA, Chiu AY, Chui LL: Inhibition of platelet-aggregating activity in thrombotic thrombocytopenic purpura plasma by normal adult immunoglobulin G. J Clin Invest 73:548, 1984

13. Deykin D, Hellerstein LJ: The assessment of drug-dependent and isoimmune antiplatelet antibodies by the use of platelet aggregometry. J Clin Invest 51:3142, 1972

14. Wu KK, Hoak JC, Thompson JS, Koepke JA: Use of platelet aggregometry in selection of compatible platelet donors. N Engl J Med 292:130, 1975

15. Hussey C, Hause L, and Gottschall J: A platelet aggregating factor in TTP: Initial activity, fluctuations, and removal by plasma exchange. Thromb Haemost 54:11, 1985

16. Kitchens CS: Studies of a patient with recurring thrombotic thrombocytopenic purpura. Am J Hematol 13:259, 1982

17. Krishnamurthy M, Bellevue R, Lian E, Dosik H: Intravenous immunoglobulin in thrombotic thrombocytopenic purpura—a new mode of treatment. Blood 66:291, 1985 (suppl 1) (abstr)

18. Moake JL, Byrnes JJ, Troll JH, Rudy CK, Weinstein MJ, Colannino NM, Hong SL: Abnormal VIII: von Willebrand factor patterns in the plasma of patients with the hemolytic-uremic syndrome. Blood 64:592, 1984

19. Byrnes JJ, Khurana M: Treatment of thrombotic thrombocytopenic purpura with plasma. N Engl J Med 297:1386, 1977

To the Editor:

We stated that von Willebrand factor (vWF) is unlikely to play a major role in the thrombotic thrombocytopenic purpura (TTP) plasma-induced platelet agglutination (or aggregation) and that glycoprotein (GP) Ib on the platelet membrane, to which vWF binds, is not involved in the development of TTP.[1] We have not excluded the involvement of vWF in the pathophysiology of TTP. However, in addition to our data, there is some evidence presently available suggesting that vWF itself is less likely to assume the key role in the pathogenesis of TTP in some or the majority of patients afflicted with this disease. Most TTP patients respond to infusion or exchange with normal whole plasma.[2,3] and, occasionally, even cryoprecipitate[4,5]; both contain vWF multimers. Contrary to the claim by Dr Moake that cryosupernatant is an effective treatment for acute episodes of TTP when other types of plasma manipulation therapy have failed, whole plasma works as well as cryosupernatant according to the literature cited.[6-8] Furthermore, following infusion with plasma in patients with chronic TTP,[6] the size of vWF was reduced for less than 24 hours; however, the remission lasted for 3 to 4 weeks. Apparently, infusion of "vWF conversion processing factor" present in the normal plasma cannot account for the prolonged remission. In clinical situations where platelet agglutination occurs as a result of hyperactive vWF or vWF receptor, such as in type IIb vWD and platelet type pseudo-vWD, typical TTP symptoms have not been reported. Murphy et al[5] described that platelet agglutinating activity appeared when large vWF multimers were markedly reduced in acute episodes of TTP. This supports our findings that vWF is not essential in TTP plasma-induced agglutination.

The observation that large vWF forms decrease or disappear from patient plasma during acute TTP episodes[4-6,9-11] is interesting and demands some explanation. It is an attractive hypothesis that large vWF multimers are cleared through their mediating platelet agglutination. However, thus far, there is neither direct in vitro nor in vivo evidence that large vWF multimers disappear during acute TTP episodes through binding to platelets. Other possibilities include the attachment of vWF to the exposed subendothelium in the damaged microvessels, decreased synthesis or release from the endothelial cells, and accelerated proteolysis of large vWF multimers due to the release of tissue plasminogen activator or other proteases during acute TTP episodes could also explain the disappearance of large vWF multimers. Our data suggested that the platelet membrane GP Ib was not involved in the platelet agglutination by plasma obtained from five TTP patients during acute episodes. We did not study whether platelet GP IIb-IIIa receptor was involved in the TTP plasma-induced platelet agglutination because the binding of vWF to the latter receptor in the activated platelets is inhibited by the physiologic level of fibrinogen in the plasma.[12] Even if vWF is consumed as a result of binding to the GP IIb-IIIa receptors exposed on the activated platelets, the question of what caused the activation of platelets would be raised. Then, the role of vWF in TTP would be secondary rather than primary. Since Dr Moake brought up the question, the effect of monoclonal antibody against GP IIb-IIIa on the TTP plasma-induced platelet agglutination was studied. It was found that the monoclonal antibody against platelet GP IIb-IIIa (kindly provided by Dr Barry Coller, Stony Brook, New York, designated as 10E5) did not inhibit the agglutination induced by the TTP plasma (unpublished data), from which the 37,000 dalton platelet agglutinating protein was purified.[13] As far as the platelet membrane mol wt 210,000 protein is concerned, its functional and structural relationship to GP Ib is not clear.[14] Whether mol wt 210,000 protein is a separate receptor for vWF remains to be clarified. Consequently, the vWF receptors on the platelet membrane are not involved in the agglutination induced by the TTP plasma containing 37,000 dalton protein. Since TTP is caused by different etiologies and pathogenetic mechanisms, our findings do not exclude entirely the possible involvement of vWF receptors on the platelet membrane in certain TTP patients.

We have previously shown that the platelet agglutination induced by three TTP plasmas is different from that induced by isoantibody because the former is inhibited by preincubation with normal adult or patient's recovery plasma, but the latter is not.[15,16] All five TTP plasma samples used in our paper[1] demonstrated such characteristics. The plasma taken from three of the five patients after recovery failed to cause platelet agglutination. The platelets procured from two patients after recovery were agglutinated by their own plasma obtained during acute disease. In addition, all five acute TTP plasma samples lost their clumping activity after incubation at 56 °C for 30 minutes, whereas the plasma with isoantibody did not (unpublished data). Furthermore, the 37,000 dalton platelet agglutinating protein (PAP) isolated from one of the TTP plasmas is definitely not an immunoglobulin.[13] This protein has been shown to be inhibited by adult human IgG and present in another two TTP plasma samples, but absent in the plasmas from normal subjects and patients with disseminated intravascular coagulation (DIC) or autoimmune thrombocytopenia.[13] This indicates that the platelet clumping substance present in the TTP plasma samples reported from our laboratory is not isoantibody. Nevertheless, we have noted that the platelet agglutination caused by some of TTP plasmas is not inhibited by normal plasma. Under these circumstances, the possibility of isoantibody-induced clumping cannot be excluded.

Plasma infusion or exchange is thought to be the mainstay of treatment for TTP. How plasma works remains to be elucidated. We have demonstrated that the IgG purified from normal adult or patient's recovery plasma inhibited the TTP plasma-induced platelet agglutination.[16] It has been postulated that IgG given through plasma infusion may contribute to the success of therapy. For the treatment of acute episodes of TTP, about 1.5 to 3 L of plasma are usually required daily. These amounts of plasma would contain about 19 to 38 g of IgG. The use of these amounts of commercial intravenous gamma globulin for acute TTP is cost-prohibitive at present. Because of the less likeliness of gamma globulin to transmit hepatitis, acquired immunodeficiency syndrome, and other infections, this treatment modality, especially the use of specific hyperimmune gamma globulin, warrants further study. In addition to IgG, there are other substances present in the normal plasma which also inhibit the TTP plasma-induced agglutination, and these are currently under our investigation. The physiologic significance of these inhibitory substances remains to be clarified. For the time being, whole plasma is recommended for the initial therapy for acute TTP.

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REFERENCES

1. Lian EC-Y, Siddiqui FA: Investigation of the role of von Willebrand factor in thrombotic thrombocytopenic purpura. Blood 66:1219, 1985

2. Byrnes JJ, Lian EC-Y: Recent therapeutic advances in thrombotic thrombocytopenic purpura. Semin Thromb Hemost 5:199, 1979

3. Bukowski RM, King JW, Hewlett JS: Plasmapheresis in the treatment of thrombotic thrombocytopenic purpura. Blood 50:413, 1977

4. Miura M, Koizumi S, Nakamura K, Ohno T, Tachinami T, Yamagami M, Taniguchi N, Kinoshita S, Abildgaard CF: Efficacy of several plasma components in a young boy with chronic thrombocytopenia and hemolytic anemia who responds repeatedly to normal plasma infusions. Am J Hematol 17:307, 1984

5. Murphy WG, Moore JC, Barr RD, Pai MKR, Kelton JG: Relationship between the presence of a platelet aggregating factor and abnormalities of factor VIII: von Willebrand's factor in a patient with chronic thrombotic thrombocytopenic purpura. Blood 66:294, 1985 (suppl 1) (abstr)

6. Moake JL, Byrnes JJ, Troll JH, Rudy CK, Hong SL, Weinstein MJ, Colannino NM: Effects of fresh-frozen plasma and its cryosupernatant fraction on von Willebrand factor multimeric forms in chronic relapsing thrombotic thrombocytopenic purpura. Blood 65:1232, 1985

7. Kitchens CS: Studies of a patient with recurring thrombotic thrombocytopenic purpura. Am J Hematol 13:259, 1982

8. Byrnes JJ, Khurana M: Treatment of thrombotic thrombocytopenic purpura with plasma. N Engl J Med 297:1386, 1977

9. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, Seder RH, Hong SL, Deykin D: Unusually large plasma factor VIII: von Willebrand and factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. N Engl J Med 307:1432, 1982

10. Rowe JM, Francis CW, Cyran EM, Marder VJ: Thrombotic thrombocytopenic purpura: Recovery after splenectomy associated with persistence of abnormally large von Willebrand factor multimers. Am J Hematol 20:161, 1985

11. Kelton JG, Moore J, Santos A, Sheridan D: The detection of a platelet agglutination factor in thrombotic thrombocytopenic purpura. Ann Intern Med 101:589, 1984

12. Pietu G, Cherel G, Marguerie G, Meyer D: Inhibition of von Willebrand factor—platelet interaction by fibrinogen. Nature 308:648, 1984

13. Siddiqui FA, Lian EC-Y: Novel platelet agglutinating protein from a thrombotic thrombocytopenic purpura plasma. J Clin Invest 76:1330, 1985

14. Stricker RB, Wong D, Saks SS, Corash L, Shuman MA: Acquired Bernard-Soulier syndrome. Evidence for the role of 210,000-molecular weight protein in the interaction of platelets with von Willebrand factor. J Clin Invest 76:1274, 1985

15. Lian EC-Y, Harkness DR, Byrnes JJ, Wallach H, Nunez R: The response of a platelet aggregating factor in the plasma of patients with thrombotic thrombocytopenic purpura and its inhibition by normal plasma. Blood 53:333, 1979

16. Lian EC-Y, Mui PTK, Siddiqui FA, Chiu AYY, Chiu LLS: Inhibition of platelet-aggregating activity in thrombotic thrombocytopenic purpura plasma by normal adult immunoglobulin G. J Clin Invest 73:548, 1984

To the Editor:

The critical review of cobalamin-folate interrelations by Chanarin et al[1] provided an excellent guide through the intricacies of the "methyl trap" hypothesis. However, our interest in patients with inborn defects in the metabolism of sulfur-containing amino acids prompts us to challenge the phrase "the maintenance of near-normal serum methionine levels in children with hereditary MTHFR deficiency" (page 486). The authors are perpetuating an error that crept into the literature and has been repeated by a widely respected reference.[2] The truth is that most patients with MTHFR deficiency have significant hypomethioninemia and the two references cited by Chanarin et al are probably unusual cases. Erbe,[3] to our knowledge, was the first to emphasize hypomethioninemia in his review of 1979, which pointed out its significance in the causation of the CNS manifestations of MTHFR deficiency. Since then other patients[4,5] with the infantile form of the disease have been described as having low blood methionine levels, although it now appears that older children and patients with a milder form of MTHFR deficiency can present with "near normal" values. We have cared for 2 infants with the disease,[6] and both had hypomethioninemia. One also had low CSF methionine levels. The other infant worsened after four and one half hours of N_2O anesthesia—before the diagnosis was made. In both, the major neuropathology was marked demyelination, which included the spinal cord. Smith et al[7] have recently described subacute combined degeneration in MTHFR deficiency. Despite the fact that infants with the disease also have homocystinemia, low 5 methyl THF, and low neurotransmitter metabolites in the CSF, we suspect that it is the CNS hypomethioninemia that is most devastating. And it is this biochemical parameter that requires early identification and energetic treatment if our dismal record in the therapy of this disorder is to improve.

Incidentally, it should be pointed out that megaloblastic anemia has not been reported in MTHFR deficiency, although the search for it has not always been rigorous.

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REFERENCES

1. Chanarin I, Deacon R, Lumb M, Muir M, Perry J: Cobalamin-folate interrelations: A critical review. Blood 66:479, 1985

2. Rowe PB: Inherited disorders of folate metabolism, in The Metabolic Basis of Inherited Disease. Stanbury JB, Syngarden JB, Fredrickson DS, Goldstein, JL, Brown MS (eds): New York, McGraw-Hill, 1983, p 516

3. Erbe RW: Congenital defects in folate metabolism. Adv Hum Genet 9:333, 1979

4. Harpey JP, Rosenblatt DS, Cooper BA, LeMoel G, Roy C, Lafourcade J: Homocystinuria caused by 5,10 methylenetetrahydrofolate reductase deficiency. J Pediatr 98:275, 1981

5. Cederbaum S, Shaw KNF, Cox DR, Erbe RW, Boss GR, Carrel RE: Pediatr Res 15:560, 1981

6. Beckman DR, Hoganson G, Berlow S, Gilbert E: Pathological findings in 5,10 methylene tetrahydrofolate reductase deficiency, in Gilbert EM, Opitz JM, Paul N (eds): Genetic Aspects of Developmental Pathology (in press)

7. Smith I, Hyland K, Kendall B, Leeming R: Clinical role of pteridine therapy in tetrahydrobiopterin deficiency. J Inherited Metab Dis 8:36, 1985 (suppl 1)

ANNOUNCEMENTS

EXECUTIVE DIRECTOR

An Executive Director is being sought for the Puget Sound Blood Center in Seattle, Washington. The PSBC is a centralized regional blood banking facility that employs 240 people, including seven physicians, and is responsible for the distribution of 250,000 units of blood components annually. Candidates must have an MD degree and have demonstrated administrative skills in transfusion medicine and the ability to contribute to and broaden the ongoing research activities of the Center. The position carries a senior faculty appointment in the School of Medicine at the University of Washington. Interested candidates should send their CV and bibliography as well as the names of three references to Dr. Daniel H. Coleman, c/o Search Committee, Puget Sound Blood Center, Terry and Madison Streets, Seattle, Washington 98104. The PSBC is an Equal Opportunity Employer.

AN INTERNATIONAL SYMPOSIUM
IN CONJUNCTION WITH THE MEDITERRANEAN SOCIETY OF THERAPY
"RECENT TRENDS IN HEPARIN THERAPY"

Wednesday, July 23, 1986 8:30 AM–6:00 PM
Ataturk Cultural Center, Istanbul, Turkey

Organizing Chairmen: Orhan T. Ulutin, Istanbul, Turkey
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INVITED SPEAKERS

M. Aiach, Hopital Broussais, Paris, France
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P. Bianchini, OPOCRIN, Corlo, Italy
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Therapeutic Monitoring of Heparin and Derivatives
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Drug Interactions With Heparin

This symposium is co-sponsored by the Mediterranean Society of Therapy, University of Istanbul, and Loyola University of Chicago, Hemostasis Research Laboratory. On Thursday, July 24, 1986 a workshop on potency evaluation, monitoring of heparin therapy, and an overview of newer methods will be held. This symposium is organized in conjunction with the Second Annual Meeting of the Mediterranean Society of Therapy, to be held in Instanbul July 20–25, 1986. Special registration arrangements will be made only for participants attending this symposium, which is accredited for eight hours of CME credit. Special group travel arrangements will be available for North American participants. For further information, please contact either of the organizing chairmen:

Dr. Jawed Fareed
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Department of Pathology, Room 2643
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Dr. Orhan N. Ulutin
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Last summer a group of biologists met concurrently with the International Society on Thrombosis and Haemostasis in San Diego, to explore possible use of electronic communication to facilitate exchange of ideas and information. This meeting was sponsored by NHLBI of the USPHS, and was targeted at the pioneer venture of establishing a free-flowing forum for scientific discussion in the field of vascular biology, hemostasis, and thrombosis, and we have been meeting and communicating since that time.

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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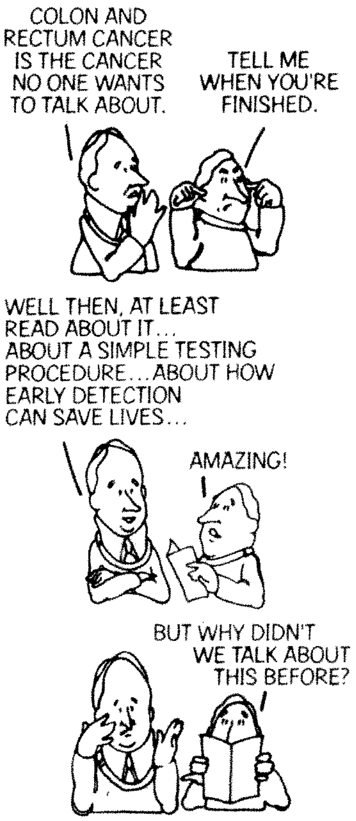
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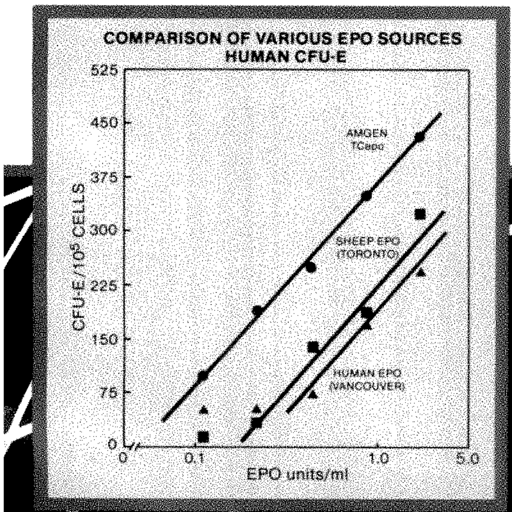

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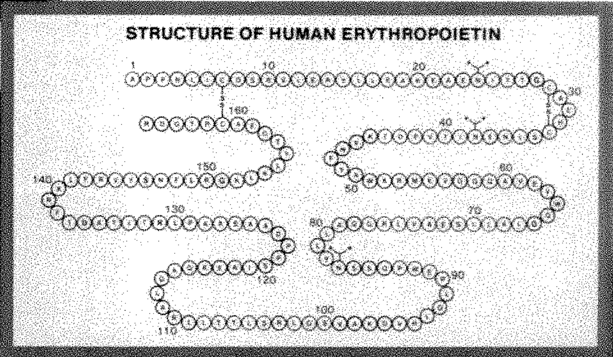

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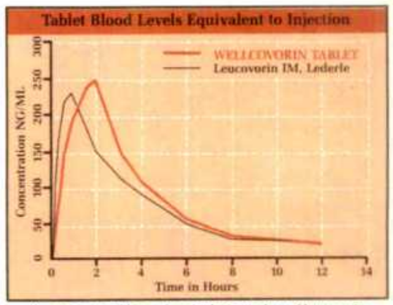
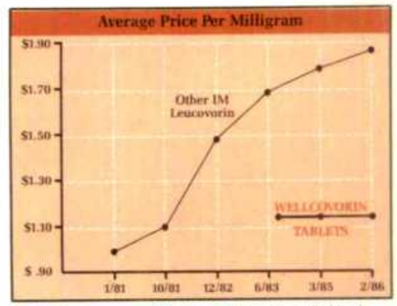


Average serum folates after single 15 mg dose of leucovorin administered either IM or orally. Adapted from Blum, et al.

Blum MR, Guaspari A, Liao SHT, et al: Bioavailability and pharmacokinetics of oral and parenteral leucovorin. 33rd National Meeting Pharm. Sci. San Diego, California. 1982. 12(2):175.



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CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12}. A hematologic remission may occur while neurologic manifestations remain progressive.

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible. As the time interval between antifolate administration (e.g. methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes.

PRECAUTIONS:

General: Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin. In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive. Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney.

Drug Interactions: Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin and primidone, and increase the frequency of seizures in susceptible children.

Pregnancy: *Teratogenic Effects:* Pregnancy Category C. Animal reproduction studies have not been conducted with Wellcovorin. It is also not known whether Wellcovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Wellcovorin should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Wellcovorin is administered to a nursing mother.

Pediatric Use: See "Drug Interactions".

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid.

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists.

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase. Leucovorin rescue must begin within 24 hours of antifolate administration. A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for seventy-two hours. If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the pre-methotrexate serum creatinine, the leucovorin dose should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M.[1,2]

The recommended dose of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (i.e. trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators.[3,4,5]

1. Bleyer WA: The Clinical Pharmacology of Methotrexate. *Cancer, 41*(1):36-51, 1978.
2. Frei E, Blum RH, Pitman SW, *et al*: High Dose Methotrexate with Leucovorin Rescue: Rational and Spectrum of Antitumor Activity. *Am J Med, 68*:370-376, 1980.
3. Golde DW, Bersch N, Quan SG: Trimethoprim and Sulpha-methoxazole Inhibition of Haematoporesis *in Vitro. Br J Haematol, 40*(3): 363-367, 1978.
4. Steinberg SE, Campbell CL, Rabinovitch PS, *et al*: The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells. *Blood, 55*(3): 501-504, 1980.
5. Mahmoud AAF and Warren KS: Algorithms in the Diagnosis and Management of Exotic Disease. XX Toxoplasmosis. *J Infect Dis, 135*(3): 493-496, 1977.

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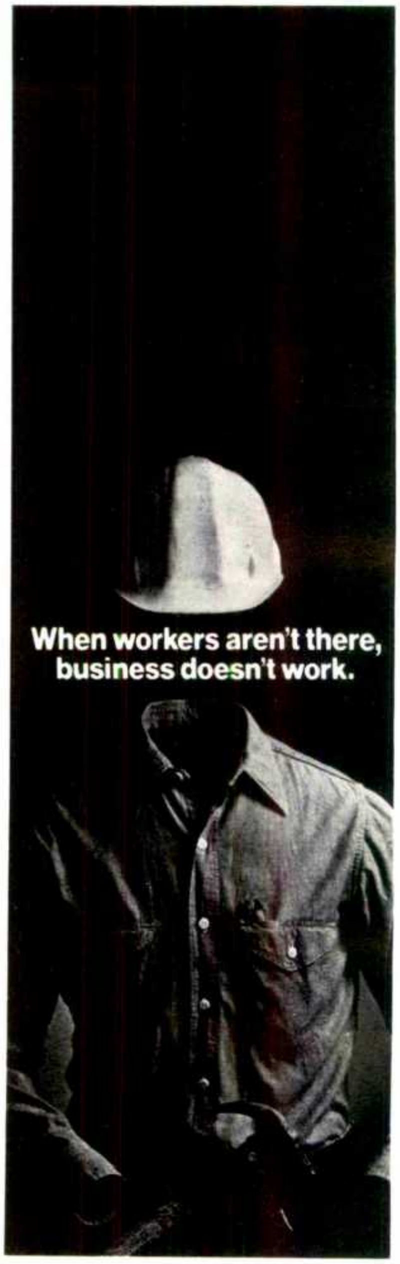

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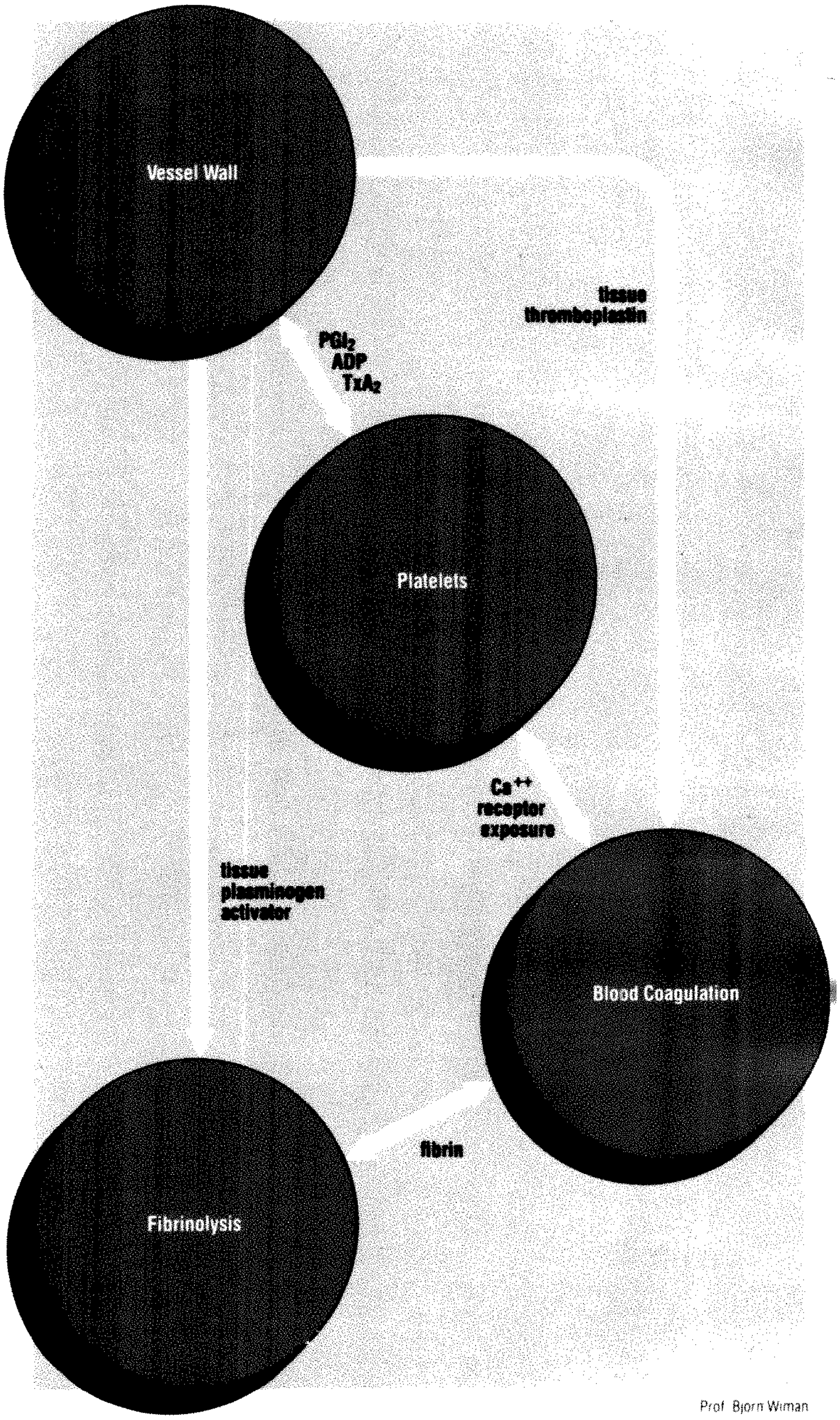

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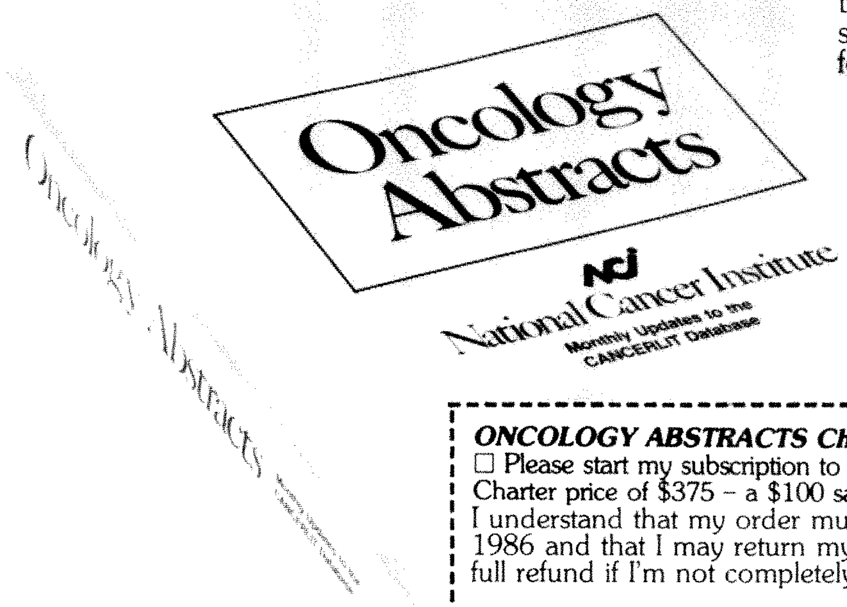
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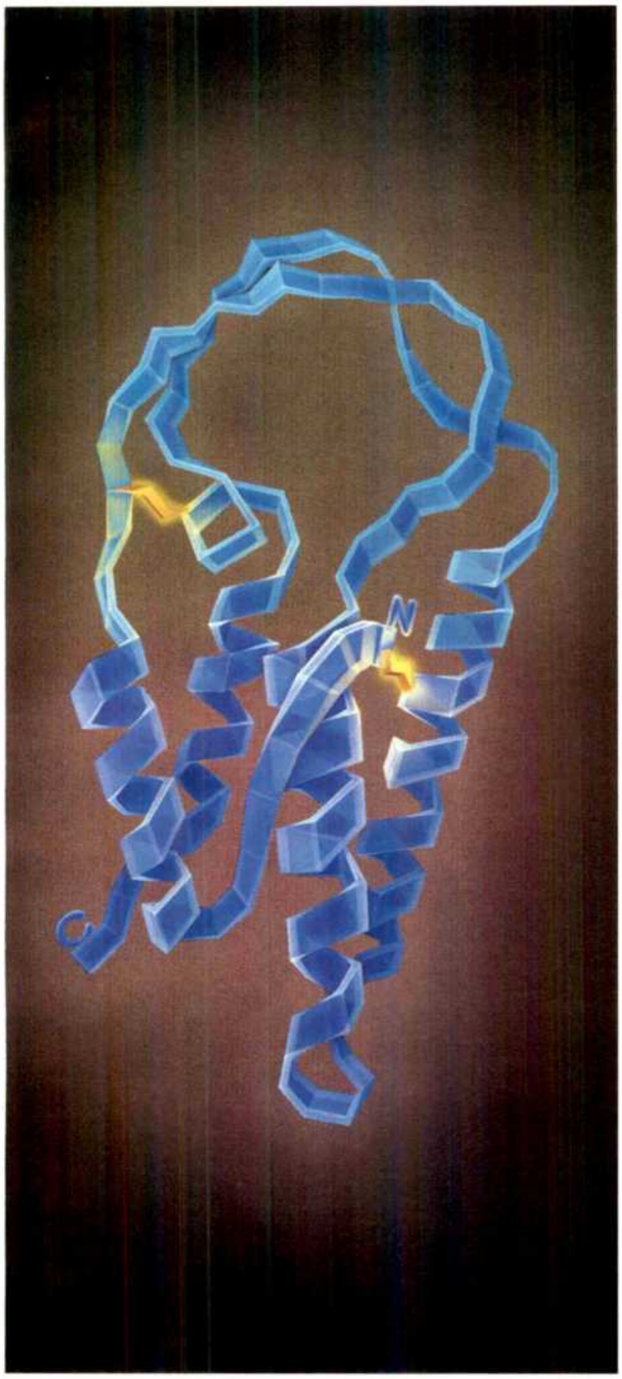

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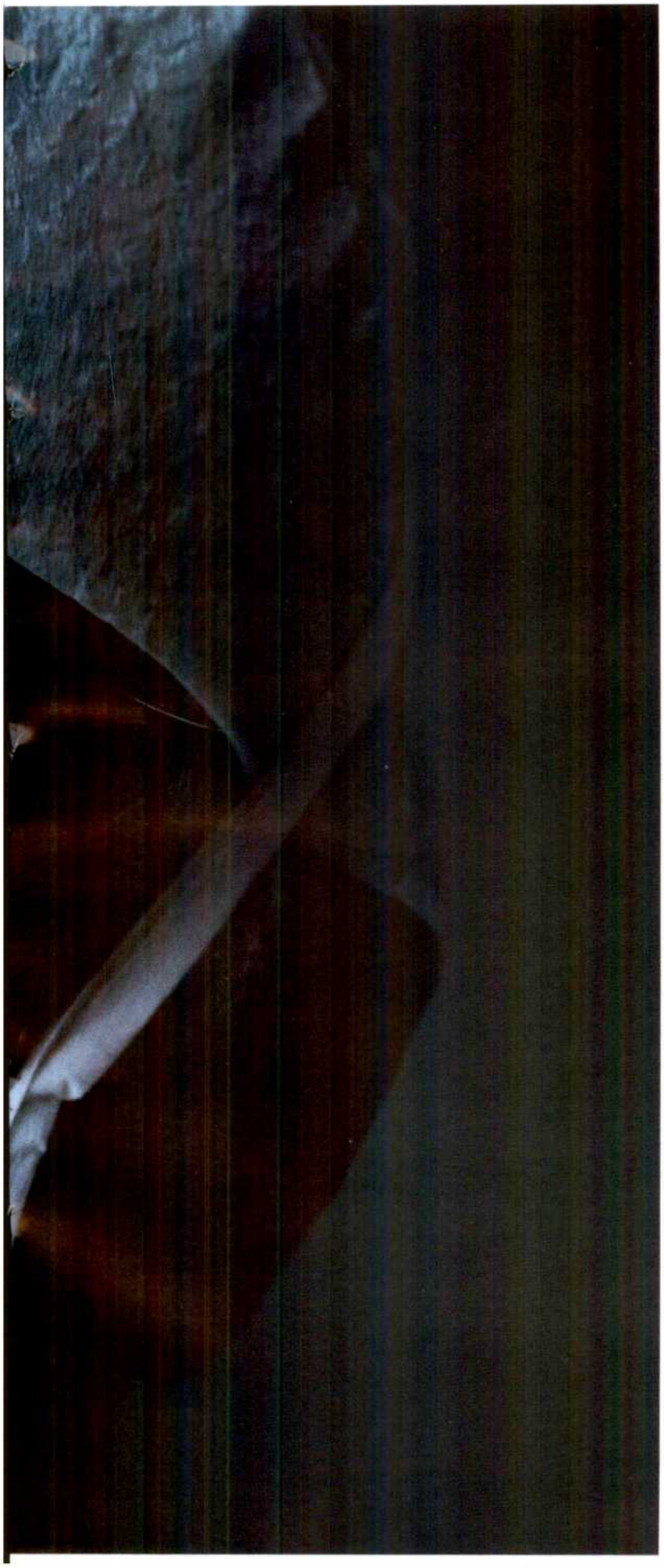

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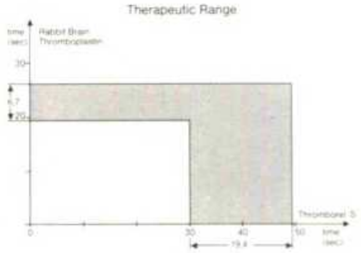

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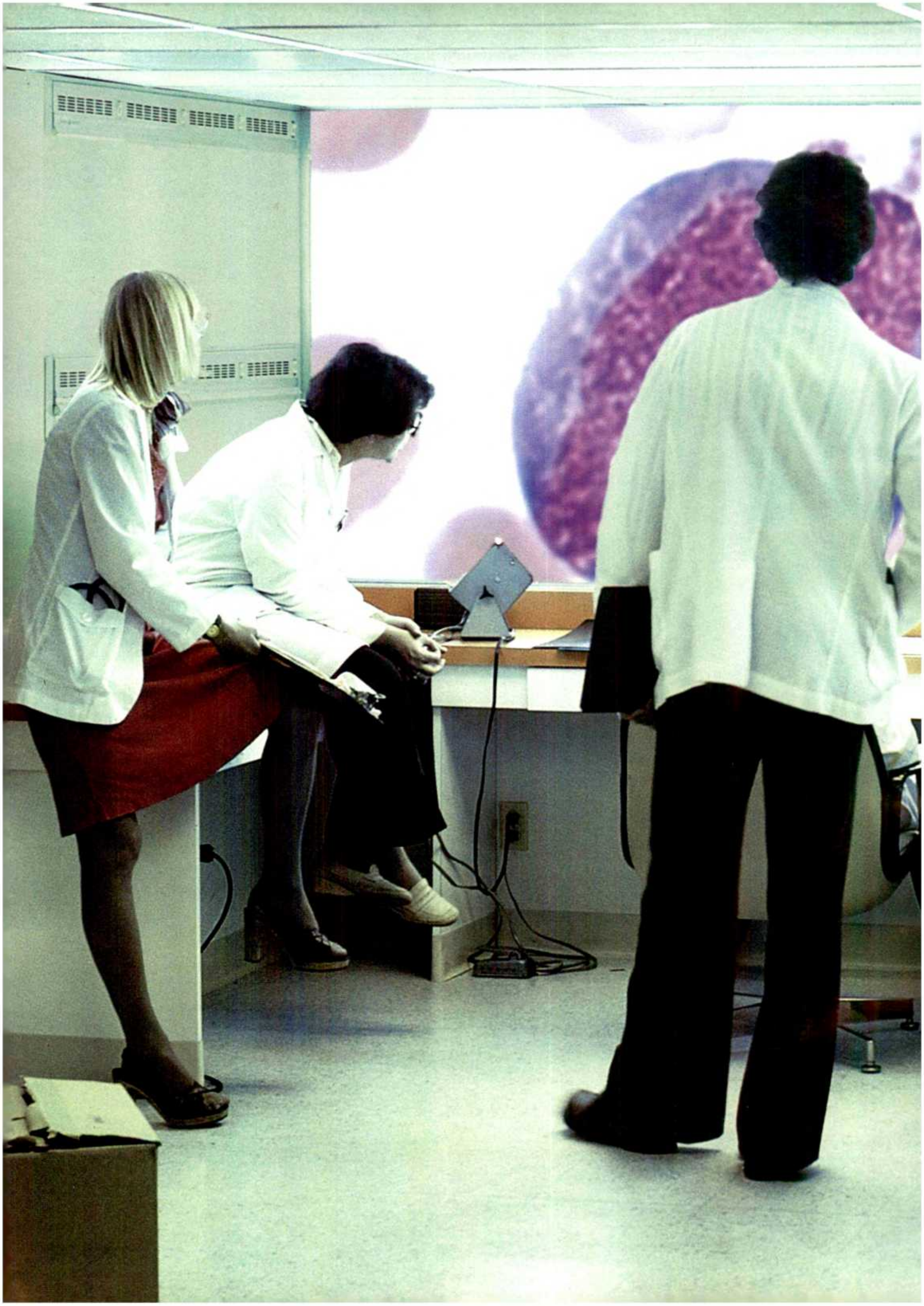

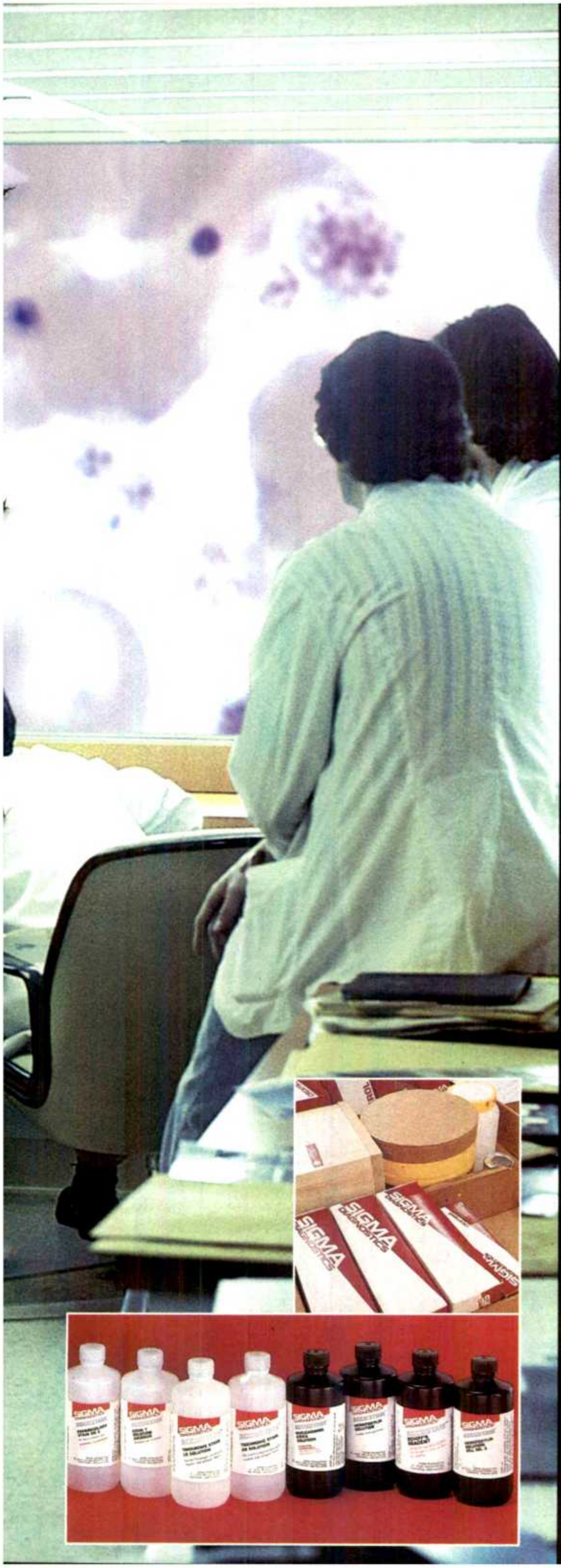

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Methotrexate††	400 mg/m²	IV	Weeks 2, 6, 10
Doxorubicin	50 mg/m²	IV	Weeks 1, 3, 5, 7, 9, 11
Cyclophosphamide	350 mg/m²	IV	Weeks 1, 3, 5, 7, 9, 11
Vincristine	1.4 mg/m²	IV	Weeks 2, 4, 6, 8, 10, 12
Bleomycin	10 U/m²	IV	Weeks 4, 8, 12
Prednisone	75 mg	Oral	Daily, dose tapered over the last 15 days
Co-trimoxazole	2 tablets	Oral	Twice daily throughout

† MACOP-B = methotrexate with leucovorin rescue, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin; IV = intravenous.

†† Methotrexate given as a 100 mg/m² intravenous bolus, then 300 mg/m² intravenously over 4 hours, followed 24 hours later by folinic acid, 15 mg orally, every 6 hours for 6 doses.

* Klimo P, Connors JM: MACOP-B chemotherapy for the treatment of diffuse large-cell lymphoma. *Ann Intern Med.* 102:596-602, 1985.

Blenoxane®

(sterile bleomycin sulfate)

Brief Summary of Prescribing Information (1) 10/84. For complete prescribing information please consult product literature.

WARNING

It is recommended that Blenoxane® be administered under the supervision of a qualified physician experienced in the use of cancer chemotherapeutic agents. Appropriate management of therapy and complications is possible only when adequate diagnostic and treatment facilities are readily available.

Pulmonary fibrosis is the most severe toxicity associated with Blenoxane. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Its occurrence is higher in elderly patients and in those receiving greater than 400 units total dose, but pulmonary toxicity has been observed in young patients and those treated with low doses.

A severe idiosyncratic reaction consisting of hypotension, mental confusion, fever, chills, and wheezing has been reported in approximately 1% of lymphoma patients treated with Blenoxane.

INDICATIONS: Blenoxane should be considered a palliative treatment. It has been shown to be useful in the management of the following neoplasms either as a single agent or in proven combinations with other approved chemotherapeutic agents:

Squamous Cell Carcinoma – Head and neck including mouth, tongue, tonsil, nasopharynx, oropharynx, sinus, palate, lip, buccal mucosa, gingiva, epiglottis, skin, larynx, penis, cervix, and vulva. The response to Blenoxane is poorer in patients with head and neck cancer previously irradiated.

Lymphomas – Hodgkin's, reticulum cell sarcoma, lymphosarcoma.

Testicular Carcinoma – Embryonal cell, choriocarcinoma, and teratocarcinoma.

CONTRAINDICATIONS: Blenoxane is contraindicated in patients who have demonstrated a hypersensitive or an idiosyncratic reaction to it.

WARNINGS: Patients receiving Blenoxane must be observed carefully and frequently during and after therapy. It should be used with extreme caution in patients with significant impairment of renal function or compromised pulmonary function.

Pulmonary toxicities occur in 10% of treated patients. In approximately 1%, the nonspecific pneumonitis induced by Blenoxane progresses to pulmonary fibrosis, and death. Although this is age and dose related, the toxicity is unpredictable. Frequent roentgenograms are recommended.

Idiosyncratic reactions similar to anaphylaxis have been reported in 1% of lymphoma patients treated with Blenoxane. Since these usually occur after the first or second dose, careful monitoring is essential after these doses.

Renal or hepatic toxicity, beginning as a deterioration in renal or liver function tests, have been reported, infrequently. These toxicities may occur, however, at any time after initiation of therapy.

Usage in Pregnancy: Safe use of Blenoxane in pregnant women has not been established.

ADVERSE REACTIONS: Pulmonary – This is potentially the most serious side effect, occurring in approximately 10% of treated patients. The most frequent presentation is pneumonitis occasionally progressing to pulmonary fibrosis. Approximately 1% of patients treated have died of pulmonary fibrosis. Pulmonary toxicity is both dose and age-related, being more common in patients over 70 years of age and in those receiving over 400 units total dose. This toxicity, however, is unpredictable and has been seen occasionally in young patients receiving low doses.

Because of lack of specificity of the clinical syndrome, the identification of patients with pulmonary toxicity due to Blenoxane has been extremely difficult. The earliest symptom associated with Blenoxane pulmonary toxicity is dyspnea. The earliest sign is fine rales.

Radiographically, Blenoxane-induced pneumonitis produces nonspecific patchy opacities, usually of lower lung fields. The most common changes in pulmonary function tests are a decrease in total lung volume and a decrease in vital capacity. However, these changes are not predictive of the development of pulmonary fibrosis.

The microscopic tissue changes due to Blenoxane toxicity include bronchiolar squamous metaplasia, reactive macrophages, atypical alveolar epithelial cells, fibrinous edema, and interstitial fibrosis. The acute stage may involve capillary changes and subsequent fibrinous exudation into alveoli producing a change similar to hyaline membrane formation and progressing to a diffuse interstitial fibrosis resembling the Hamman-Rich syndrome. These microscopic findings are nonspecific, e.g., similar changes are seen in radiation pneumonitis, pneumocystic pneumonitis.

To monitor the onset of pulmonary toxicity, roentgenograms of the chest should be taken every 1 to 2 weeks. If pulmonary changes are noted, treatment should be discontinued until it can be determined if they are drug related. Recent studies have suggested that sequential measurement of the pulmonary diffusion capacity for carbon monoxide (DL_{CO}) during treatment with Blenoxane may be an indicator of subclinical pulmonary toxicity. It is recommended that the DL_{CO} be monitored monthly if it is to be employed to detect pulmonary toxicities, and thus the drug should be discontinued when the DL_{CO} falls below 30 to 35% of the pretreatment value.

Because of bleomycin's sensitization of lung tissue, patients who have received bleomycin are at greater risk of developing pulmonary toxicity when oxygen is administered at surgery. While long exposure to very high oxygen concentrations is a known cause of lung damage, after bleomycin administration, lung damage can occur at lower concentrations than usually would be considered safe. Suggested preventive measures are:

(1) Maintain FI O_2 at concentrations approximately that of room air (25%) during surgery and the post-operative period.

(2) Monitor carefully fluid replacement, focusing more on colloid administration rather than crystalloid.

Idiosyncratic Reactions – In approximately 1% of the lymphoma patients treated with Blenoxane an idiosyncratic reaction, similar to anaphylaxis clinically, has been reported. The reaction may be immediate or delayed for several hours, and usually occurs after the first or second dose. It consists of hypotension, mental confusion, fever, chills, and wheezing. Treatment is symptomatic including volume expansion, pressor agents, antihistamines, and corticosteroids.

Integument and Mucous Membranes – These are the most frequent side effects, being reported in approximately 50% of treated patients. These consist of erythema, rash, striae, vesiculation, hyperpigmentation, and tenderness of the skin. Hyperkeratosis, nail changes, alopecia, pruritus, and stomatitis have also been reported. It was necessary to discontinue Blenoxane therapy in 2% of treated patients because of these toxicities.

Skin toxicity is a relatively late manifestation usually developing in the 2nd and 3rd week of treatment after 150 to 200 units of Blenoxane have been administered and appears to be related to the cumulative dose.

Other – Fever, chills, and vomiting were frequently reported side effects. Anorexia and weight loss are common and may persist long after termination of the medication. Pain at tumor site, phlebitis, and other local reactions were reported infrequently.

There are isolated reports of Raynaud's phenomenon occurring in patients with testicular carcinomas treated with a combination of Blenoxane and Velban®. It is currently unknown if the cause for the Raynaud's phenomenon in these cases is the disease, Blenoxane, Velban, or a combination of any or all of these.

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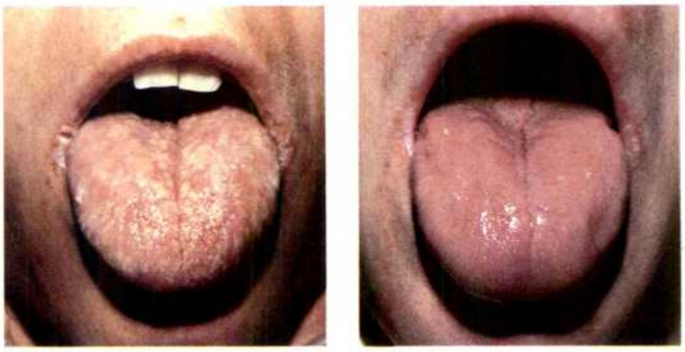

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A clinical cure rate of 96% was achieved in one randomized double-blind trial[2] in which MYCELEX Troche was administered to patients with oropharyngeal candidiasis. Other investigators have concluded that MYCELEX Troche "is highly effective treatment for chronic oral candidiasis."[3]

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*Clinical cure is defined as absence of signs and symptoms of the disease.

†See adjacent page for summary of prescribing information, including contraindications and adverse reactions.

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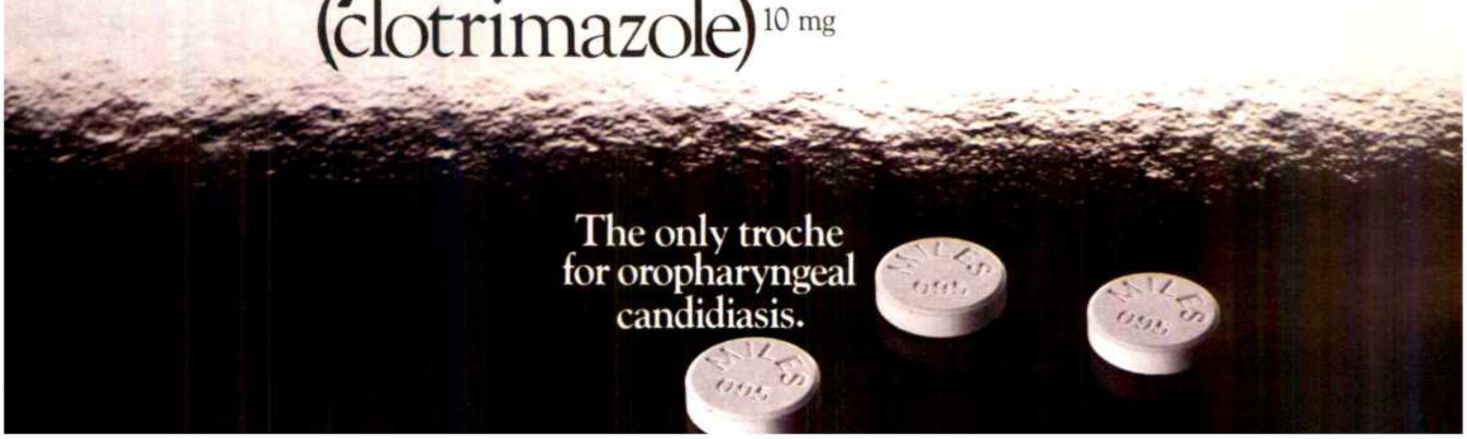
(clotrimazole) 10 mg



MYCELEX® (clotrimazole) TROCHE

Indications and Usage: Mycelex Troches are indicated for the local treatment of oropharyngeal candidiasis. The diagnosis should be confirmed by a KOH smear and/or culture prior to treatment.

Contraindications: Mycelex Troches are contraindicated in patients who are hypersensitive to any of the components.

Warning: Mycelex Troches are not indicated for the treatment of systemic mycoses.

Precautions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials. In most cases the elevations were minimal and it was often impossible to distinguish effects of clotrimazole from those of other therapy and the underlying disease (malignancy in most cases). Periodic assessment of hepatic function is advisable particularly in patients with preexisting hepatic impairment.

Since patients must be instructed to allow each troche to dissolve slowly in the mouth in order to achieve maximum effect of the medication, they must be of such an age and physical and/or mental condition to comprehend such instructions.

Carcinogenesis: An 18-month dosing study with clotrimazole in rats has not revealed any carcinogenic effect.

Usage in Pregnancy: Pregnancy Category C: Clotrimazole has been shown to be embryotoxic in rats and mice when given in doses 100 times the adult human dose (in mg/kg), possibly secondary to maternal toxicity. The drug was not teratogenic in mice, rabbits, and rats when given in doses up to 200, 180, and 100 times the human dose.

Clotrimazole given orally to mice from nine weeks before mating through weaning at a dose 120 times the human dose was associated with impairment of mating, decreased number of viable young, and decreased survival to weaning. No effects were observed at 60 times the human dose. When the drug was given to rats during a similar time period at 50 times the human dose, there was a slight decrease in the number of pups per litter and decreased pup viability.

There are no adequate and well-controlled studies in pregnant women. Clotrimazole troches should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Pediatric Use: Safety and effectiveness of clotrimazole in children below the age of three years have not been established; therefore, its use in such patients is not recommended.

Adverse Reactions: Abnormal liver function tests have been reported in patients treated with clotrimazole troches; elevated SGOT levels were reported in about 15% of patients in the clinical trials (see Precautions section).

Nausea and vomiting was reported in about one in 20 patients.

Dosage and Administration: Mycelex Troches are administered only as a lozenge that must be dissolved slowly in the mouth. The recommended dose is one troche five times a day for 14 consecutive days. Only limited data are available on the safety and effectiveness of the clotrimazole troche after prolonged administration; therefore, therapy should be limited to short-term use, if possible.

How Supplied: Mycelex Troches—white, discoid, uncoated tablets are supplied in bottles of 70 and 140. Each tablet will be identified with the following: Miles 995.

Store below 86°F (30°C), avoid freezing.

References: 1. Montes LF, et al: Clotrimazole troches: a new therapeutic approach to oral candidiasis. *Cutis* 17:277-280, 1976. **2.** Yap B-S, Bodey GP: Oropharyngeal candidiasis treated with a troche form of clotrimazole. *Arch Intern Med* 139:656-657, 1979. **3.** Kirkpatrick CH, Alling DW: Treatment of chronic oral candidiasis with clotrimazole troches: a controlled clinical trial. *N Engl J Med* 299:1201-1203, 1978. **4.** Data on file, Miles Pharmaceuticals.

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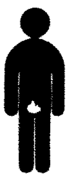
SIX WARNING SIGNS OF KIDNEY RELATED DISEASES

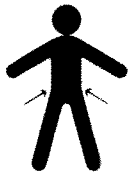


Burning or difficulty during urination

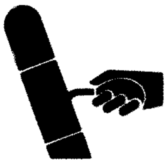
More frequent urination, particularly at night

Passage of bloody-appearing urine

Puffiness around eyes, swelling of hands and feet, especially in children



Pain in small of back just below the ribs (not aggravated by movement)



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HERPES SIMPLEX INFECTION IN THE COMPROMISED HOST:

OFTEN SERIOUS, TOO OFTEN UNSUSPECTED

Number one in a series of typical case presentations.

Esophagitis in a leukemia patient

In patients with hematologic malignancies, the visceral organ most frequently involved by herpes simplex virus (HSV) infection is the esophagus.[1] Extensive ulcerative lesions may develop on the upper two thirds of the esophagus with confluent lesions of the distal third.[2] In some cases, however, the mucosa may be grossly unremarkable even when microscopic examination shows evidence of herpetic ulcerative esophagitis.[1] Similarly, some patients may display symptoms of dysphagia and burning retrosternal pain,[3] whereas others may appear asymptomatic.[1] The diagnostic method of choice for patients with suspected herpetic esophagitis is esophagoscopy with visualization of the mucosa and procurement of cytologic and biopsy specimens from ulcerated areas.[1]

References: 1. Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **2.** Wong KK, Hirsch MS: Herpes virus infections in patients with neoplastic disease. Diagnosis and therapy. *Am J Med* 1984; 76:464-478. **3.** Lightdale CJ, Wolf DJ, Marcucci RA, et al: Herpetic esophagitis in patients with cancer: Ante mortem diagnosis by brush cytology. *Cancer* 1977;39:223-226.

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Herpetic infections may result in extensive disease and death

Immunocompromised patients are highly vulnerable to HSV infection. Such infections may interrupt therapy and even lead to death if not detected early and managed aggressively.[1] Although the initial appearance may be mild, HSV infection may progress to severe localized mucocutaneous ulcerations. The greatest threat that herpes simplex poses is an infection that disseminates from a mucocutaneous or unrecognized primary lesion and involves the lungs, liver, esophagus, or central nervous system.[1] Furthermore, herpetic ulcers frequently provide an entry for secondary bacterial and fungal pathogens.[2]

Infections may not be diagnosed correctly without virologic study

Some investigators claim that the actual incidence of serious HSV infection is probably higher than reported because "the possibility of herpetic disease is insufficiently considered and appropriate diagnostic viral studies are often not carried out."[3] One study, for example, found histocytologic evidence of visceral herpes infection in 56 patients even though herpetic involvement was not suspected in any of the patients prior to autopsy.[2]

ZOVIRAX I.V. halts viral replication and speeds healing even in debilitated patients

ZOVIRAX I.V. has been shown to be a highly effective treatment for severe mucocutaneous HSV infection in immunocompromised patients. In controlled trials, ZOVIRAX I.V. rapidly halted viral shedding — within 3 days in most patients — and significantly shortened healing time as well as duration of pain.[4,5] Furthermore, ZOVIRAX I.V. may allow patients to return home sooner and enable patients with intraoral lesions to resume normal eating habits.[6]

ZOVIRAX I.V. is well tolerated by immunocompromised patients

A multicenter collaborative trial of ZOVIRAX I.V. in immunocompromised patients reported that: "Clinical benefits were obtained without appreciable toxicity. The most common reactions were irritation of peripheral veins used for drug infusion and a low incidence of rash.... Significant renal or hematologic toxicity did not occur, even in these seriously ill patients who were commonly receiving other drugs concomitantly."[5]

Note: Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes. Please see brief summary for more information.

References: **1.** Feldman S, Cox F: Viral infections and hematological malignancies. *Clin Haematol* 1976;5:311-328. **2.** Buss DH, Scharyj M: Herpesvirus infection of the esophagus and other visceral organs in adults. Incidence and clinical significance. *Am J Med* 1979;66:457-462. **3.** Muller SA, Herrmann EC, Winkelmann RK: Herpes simplex infections in hematologic malignancies. *Am J Med* 1972;52:102-114. **4.** Wade JC, Newton B, McLaren C, et al: Intravenous acyclovir to treat mucocutaneous herpes simplex virus infection after marrow transplantation. *Ann Intern Med* 1982;96:265-269. **5.** Meyers JD, Wade JC, Mitchell CD, et al: Multicenter collaborative trial of intravenous acyclovir for treatment of mucocutaneous herpes simplex virus infection in the immunocompromised host. *Am J Med* 1982;73(1A):229-235. **6.** Data on file, Burroughs Wellcome Co.

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ZOVIRAX® I.V. INFUSION (acyclovir sodium) sterile powder

FOR INTRAVENOUS INFUSION ONLY

INDICATIONS AND USAGE: Zovirax Sterile Powder is indicated for the treatment of initial and recurrent mucosal and cutaneous Herpes simplex (HSV-1 and HSV-2) infections in immunocompromised adults and children. It is also indicated for severe initial clinical episodes of herpes genitalis in patients who are not immunocompromised.

These indications are based on the results of several double-blind, placebo-controlled studies which evaluated the drug's effect on virus excretion, complete healing of lesions, and relief of pain.

Herpes Simplex Infections in Immunocompromised Patients

A multicenter trial of Zovirax Sterile Powder at a dose of 250 mg/M^2 every 8 hours (750 mg/M^2/day) for 7 days was conducted in 97 immunocompromised patients with oro-facial, esophageal, genital and other localized infections (50 treated with Zovirax and 47 with placebo). Zovirax significantly decreased virus excretion, reduced pain, and promoted scabbing and rapid healing of lesions.[1,2,3]

Initial Episodes of Herpes Genitalis

A controlled trial was conducted in 28 patients with severe initial episodes of herpes genitalis with a Zovirax dosage of 5 mg/kg every 8 hours for 5 days (12 patients treated with Zovirax and 16 with placebo). Significant treatment effects were seen in elimination of virus from lesions and in reduction of healing times.[4]

In a similar study, 15 patients with initial episodes of genital herpes were treated with Zovirax 5 mg/kg every 8 hours for 5 days and 15 with placebo. Zovirax decreased the duration of viral excretion, new lesion formation, duration of vesicles and promoted more rapid healing of all lesions.[5]

Diagnosis

The use of appropriate laboratory diagnostic procedures will help to establish the etiologic diagnosis. Positive cultures for Herpes simplex virus offer a reliable means for confirmation of the diagnosis. In initial episodes of genital herpes, appropriate examinations should be performed to rule out other sexually transmitted diseases. Whereas cutaneous lesions associated with Herpes simplex infections are often characteristic, the finding of multinucleated giant cells in smears prepared from lesion exudate or scrapings may assist in the diagnosis.[6]

CONTRAINDICATIONS: Zovirax Sterile Powder is contraindicated for patients who develop hypersensitivity to the drug

WARNINGS: Zovirax Sterile Powder is intended for intravenous infusion only, and should not be administered topically, intramuscularly, orally, subcutaneously, or in the eye. Intravenous infusions must be given over a period of at least 1 (one) hour to prevent renal tubular damage (see PRECAUTIONS and DOSAGE AND ADMINISTRATION).

PRECAUTIONS:

General: The recommended dosage, frequency and length of treatment should not be exceeded (See DOSAGE AND ADMINISTRATION).

Although the aqueous solubility of acyclovir sodium (for infusion) is >100 mg/ml, precipitation of acyclovir crystals in renal tubules can occur if the maximum solubility of free acyclovir (2.5 mg/ml at 37°C in water) is exceeded or if the drug is administered by bolus injection. This complication causes a rise in serum creatinine and blood urea nitrogen (BUN), and a decrease in renal creatinine clearance. Ensuing renal tubular damage can produce acute renal failure.

Abnormal renal function (decreased creatinine clearance) can occur as a result of acyclovir administration and depends on the state of the patient's hydration, other treatments, and the rate of drug administration. Bolus administration of the drug leads to a 10% incidence of renal dysfunction, while in controlled studies, infusion of 5 mg/kg (250 mg/M^2) over an hour was associated with a lower frequency — 4.6%. Concomitant use of other nephrotoxic drugs, pre-existing renal disease, and dehydration make further renal impairment with acyclovir more likely. In most instances, alterations of renal function were transient and resolved spontaneously or with improvement of water and electrolyte balance, drug dosage adjustment or discontinuation of drug administration. However, in some instances, these changes may progress to acute renal failure.

Administration of Zovirax by intravenous infusion must be accompanied by adequate hydration. Since maximum urine concentration occurs within the first 2 hours following infusion, particular attention should be given to establishing sufficient urine flow during that period in order to prevent precipitation in renal tubules.

When dosage adjustments are required they should be based on estimated creatinine clearance (See DOSAGE AND ADMINISTRATION).

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma. Zovirax should be used with caution in those patients who have underlying neurologic abnormalities and those with serious renal, hepatic, or electrolyte abnormalities or significant hypoxia. It should also be used with caution in patients who have manifested prior neurologic reactions to cytotoxic drugs or those receiving concomitant intrathecal methotrexate or interferon.

Exposure of HSV isolates to acyclovir *in vitro* can lead to the emergence of less sensitive viruses. These viruses usually are deficient in thymidine kinase (required for acyclovir activation) and are less pathogenic in animals. Similar isolates have been observed in 6 severely immunocompromised patients during the course of controlled and uncontrolled studies of intravenously administered Zovirax. These occurred in patients with congenital severe combined immunodeficiencies or following bone marrow transplantation. The presence of these viruses was not associated with a worsening of clinical illness and, in some instances, the virus disappeared spontaneously. The possibility of the appearance of less sensitive viruses must be borne in mind when treating such patients. The relationship between the *in vitro* sensitivity of herpesviruses to acyclovir and clinical response to therapy has yet to be established.

Drug Interactions: Co-administration of probenecid with acyclovir has been shown to increase the mean half-life and the area under the concentration-time curve. Urinary excretion and renal clearance were correspondingly reduced. Clinical experience has identified no other significant interactions resulting from administration of other drugs concomitantly with Zovirax Sterile Powder.

Carcinogenesis, Mutagenesis, Impairment of Fertility: Acyclovir was tested in lifetime bioassays in rats and mice at single daily doses of 50, 150 and 450 mg/kg given by gavage. There was no statistically significant difference in the incidence of tumors between treated and control animals, nor did acyclovir appear to shorten the latency of tumors. In 2 *in vitro* cell transformation assays, used to provide preliminary assessment of potential oncogenicity in advance of these more definitive lifetime bioassays in rodents, conflicting results were obtained. Acyclovir was positive at the highest dose used in one system and the resulting morphologically transformed cells formed tumors when inoculated into immunosuppressed, syngeneic, weanling mice. Acyclovir was negative in another transformation system.

No chromosome damage was observed at maximum tolerated parenteral doses of 100 mg/kg acyclovir in rats or Chinese hamsters; higher doses of 500 and 1000 mg/kg were clastogenic in Chinese hamsters. In addition, no activity was found in a dominant lethal study in mice. In 9 of 11 microbial and mammalian cell assays, no evidence of mutagenicity was observed. In 2 mammalian cell assays (human lymphocytes and L5178Y mouse lymphoma cells *in vitro*), positive responses for mutagenicity and chromosomal damage occurred, but only at concentrations at least 25 times the acyclovir plasma levels achieved in man.

Acyclovir does not impair fertility or reproduction in mice at oral doses up to 450 mg/kg/day. In female rabbits treated subcutaneously with acyclovir subsequent to mating, there was a statistically significant decrease in implantation efficiency but no concomitant decrease in litter size at a dose of 50 mg/kg/day.

Pregnancy: Teratogenic Effects. Pregnancy Category C. Acyclovir was not teratogenic in the mouse (450 mg/kg/day, p.o.), rabbit (50 mg/kg/day, s.c.) or rat (50 mg/kg/day, s.c.).

Although maximum tolerated doses were tested in teratology studies, the plasma levels obtained did not exaggerate maximum plasma levels that might occur with clinical use of intravenous acyclovir.

There have been no adequate and well-controlled studies in pregnant women. Acyclovir should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Zovirax is administered to a nursing woman.

ADVERSE REACTIONS: The most frequent adverse reactions reported during controlled clinical trials of Zovirax in 64 patients were inflammation or phlebitis at the injection site following infiltration of the I.V. fluid in 9 (14.0%), transient elevations of serum creatinine in 3 (4.7%), and rash or hives in 3 (4.7%). Less frequent adverse reactions were diaphoresis, hematuria, hypotension, headache and nausea, each of which occurred in 1 patient (1.6%). Of the 63 patients receiving placebo, 3 (4.8%) experienced inflammation/phlebitis and 3 (4.8%) experienced rash or itching. Hematuria and nausea were experienced by placebo recipients at the same frequency.

Among 51 immunocompromised patients, one, a bone marrow transplant recipient with pneumonitis, developed seizures, cerebral edema, coma and expired with changes consistent with cerebral anoxia on postmortem biopsy; another immunocompromised patient exhibited coarse tremor and clonus.

Additional adverse reactions were reported in uncontrolled trials. The most frequent adverse reaction was elevated serum creatinine. This occurred in 9.8 percent of patients, usually following rapid (less than 10 minutes) intravenous infusion. Less frequent adverse experiences were thrombocytosis and jitters, each in 0.4% of patients.

Approximately 1% of patients receiving intravenous acyclovir have manifested encephalopathic changes characterized by either lethargy, obtundation, tremors, confusion, hallucinations, agitation, seizures or coma (see PRECAUTIONS).

OVERDOSAGE: Overdosage has been reported following administration of bolus injections, or inappropriately high doses, and in patients whose fluid and electrolyte balance was not properly monitored. This has resulted in elevations in BUN, serum creatinine and subsequent renal failure.

Precipitation of acyclovir in renal tubules may occur when the solubility (2.5 mg/ml) in the intratubular fluid is exceeded (see PRECAUTIONS). A six hour hemodialysis results in a 60% decrease in plasma acyclovir concentration. Data concerning peritoneal dialysis are incomplete but indicate that this method may be significantly less efficient in removing acyclovir from the blood. In the event of acute renal failure and anuria, the patient may benefit from hemodialysis until renal function is restored (see DOSAGE AND ADMINISTRATION).

DOSAGE AND ADMINISTRATION: CAUTION — RAPID OR BOLUS INTRAVENOUS AND INTRAMUSCULAR OR SUBCUTANEOUS INJECTION MUST BE AVOIDED.

Dosage: ***MUCOSAL AND CUTANEOUS HERPES SIMPLEX (HSV-1 and HSV-2) INFECTIONS IN IMMUNOCOMPROMISED PATIENTS*** — 5 mg/kg infused at a constant rate over 1 hour, every 8 hours (15 mg/kg/day) for 7 days in adult patients with normal renal function. In children under 12 years of age, more accurate dosing can be attained by infusing 250 mg/M^2 at a constant rate over 1 hour, every 8 hours (750 mg/M^2/day) for 7 days.

SEVERE INITIAL CLINICAL EPISODES OF HERPES GENITALIS — The same dose given above — administered for 5 days

Therapy should be initiated as early as possible following onset of signs and symptoms.

PATIENTS WITH ACUTE OR CHRONIC RENAL IMPAIRMENT: Refer to DOSAGE AND ADMINISTRATION section for recommended doses, and adjust the dosing interval as indicated in the table below.

Creatinine Clearance (ml/min/1.73M^2)	Dose (mg/kg)	Dosing Interval (hours)
>50	5	8
25-50	5	12
10-25	5	24
0-10	2.5	24

Hemodialysis: For patients who require dialysis, the mean plasma half-life of acyclovir during hemodialysis is approximately 5 hours. This results in a 60% decrease in plasma concentrations following a 6 hour dialysis period. Therefore, the patient's dosing schedule should be adjusted so that a dose is administered after each dialysis.

Method of Preparation: Each 10 ml vial contains acyclovir sodium equivalent to 500 mg of acyclovir. The contents of the vial should be dissolved in 10 ml of sterile water for injection or bacteriostatic water for injection containing benzyl alcohol yielding a final concentration of 50 mg/ml of acyclovir (pH approximately 11). Shake the vial well to assure complete dissolution before measuring and transferring each individual dose. DO NOT USE BACTERIOSTATIC WATER FOR INJECTION CONTAINING PARABENS. It is incompatible with Zovirax Sterile Powder and may cause precipitation.

References: **1.** C.D. Mitchell, *et al.*, Lancet *1*(8235): 1389-1392, Jun. 27, 1981. **2.** J.C. Wade, *et al.*, Ann. Intern. Med. *96*(3): 265-269, Mar. 1982. **3.** J.D. Meyers, *et al.*, Am. J. Med. 73(1A): 229-235, Jul. 20, 1982. **4.** Data on file, Burroughs Wellcome Co. **5.** A. Mindel, *et al.*, Lancet *1*(8274): 697-700, Mar. 27, 1982. **6.** Z.M. Naib, *et al.*, Cancer Res. 33:1452-1463, 1973.

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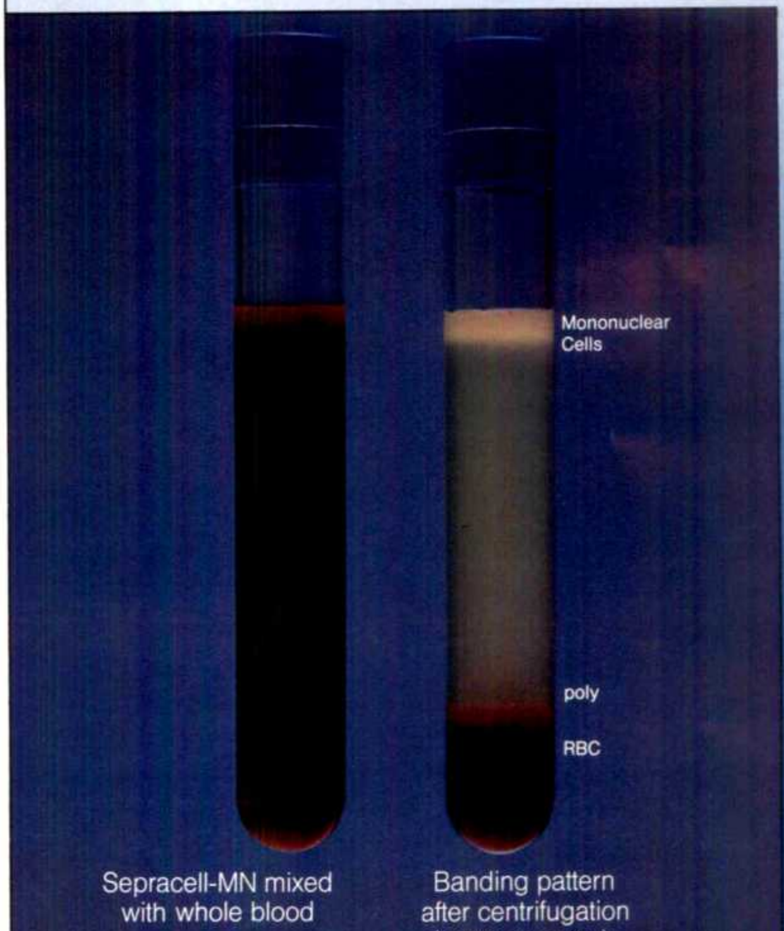

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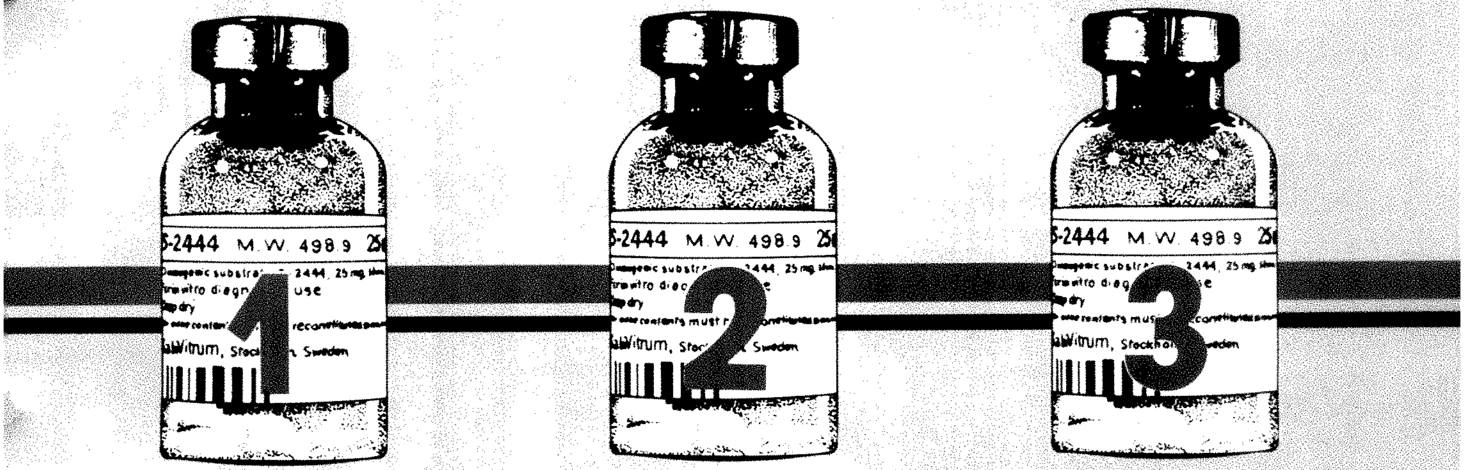

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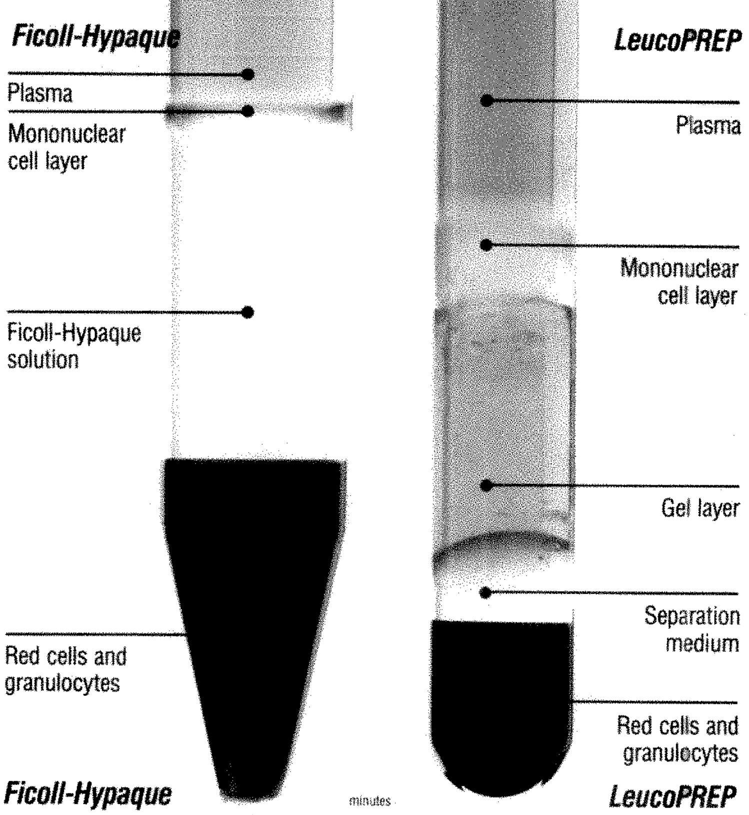

Ficoll-Hypaque	minutes		***LeucoPREP***
Dilute whole blood	6	0	No dilution or set-up required
Pipette Ficoll-Hypaque into centrifuge tube	6		
Carefully layer blood sample onto Ficoll-Hypaque	18	6	Pipette whole blood into LeucoPREP Tube
Centrifuge	35	10-15	Centrifuge
Total Time Required	***65***	***16-21***	***Total Time Required***

Total Labor Savings with LeucoPREP—44 to 49 minutes!

Comparison based on preparation of six samples. There is no significant difference in yield or purity between the two methods. Data available on request.

Sterile interior. For *in vitro* diagnostic use. Not intended for blood collection.

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Announcing...
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Leucovorin Calcium Tablets 5mg in the first Unit of Issue package

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Please see brief summary
of prescribing information
on next page.

Hope... Through Research™ from LEDERLE LABORATORIES

LEUCOVORIN CALCIUM

LEUCOVORIN CALCIUM INJECTION
Military Depot: NSN 6505-01-054-7008,
50 mg/vial for I.M. use

DESCRIPTION: Each 1 ml ampul of Leucovorin Calcium *Lederle* contains Leucovorin 3 mg as the calcium salt which is the form preferred for intramuscular injection Preservative benzyl alcohol 0.9% w/v The inactive ingredients are sodium chloride 0 56% w/v. Water for Injection qs 100% and sodium hydroxide or hydrochloric acid is used to adjust the pH to approximately 7 7

Each 50 mg vial of Leucovorin Calcium cryodesiccated powder when reconstituted with 5 ml of sterile diluent contains Leucovorin 10 mg per ml as the calcium salt which is the form preferred for intramuscular injection Contains no preservative. Dilute only with Bacteriostatic Water for Injection USP which contains benzyl alcohol The inactive ingredients are sodium chloride 40 mg/vial, and sodium hydroxide or hydrochloric acid qs to pH approximately 8 1 When reconstituted as directed, the resulting solution must be used within seven days If the product is reconstituted with Water for Injection USP, use immediately

ACTIONS: Leucovorin (folinic acid) is the formyl derivative and active form of folic acid

Leucovorin Calcium is useful clinically in circumventing the action of folate reductase There is no evidence that intramuscular doses of greater than 1 mg have greater efficacy than those of 1 mg.

INDICATIONS: Indicated (a) to diminish the toxicity and counteract the effect of inadvertently administered overdosages of folic acid antagonists (See Warnings) (b) In the treatment of the megaloblastic anemias due to sprue, nutritional deficiency, pregnancy and infancy when oral therapy is not feasible

CONTRAINDICATIONS: Not to be administered for the treatment of pernicious anemia or other megaloblastic anemias where Vitamin B_{12} is deficient

WARNINGS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to lack of Vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

In the treatment of overdosage of folic acid antagonists, Leucovorin must be administered within one hour, if possible, and is usually ineffective if administered after a delay of four hours.

PRECAUTIONS: In the presence of pernicious anemia a hematologic remission may occur while neurologic manifestations remain progressive

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

DOSAGE: Megaloblastic anemia No more than or up to 1 mg daily There is no evidence that intramuscular doses greater than 1 mg daily have greater efficacy than those of 1 mg, additionally, loss of folate in the urine becomes roughly logarithmic as the amount administered exceeds 1 mg

For the treatment of overdosage of folic acid antagonists To be given in amounts equal to the weight of the antagonist given

HOW SUPPLIED:

NDC 0205-4004-51 — 6 — 1 ml Ampuls
NDC 0205-5330-92 — 50 mg Vial of Cryodesiccated powder

LEDERLE PARENTERALS, INC
Carolina, Puerto Rico 00630 Rev 11/80

LEUCOVORIN CALCIUM TABLETS

BRIEF SUMMARY

Before prescribing Leucovorin Calcium Tablets, please consult complete prescribing information

INDICATIONS AND USAGE: Leucovorin Calcium Tablets are indicated for the prophylaxis and treatment of undesired hematopoietic effects of folic acid antagonists (see WARNINGS)

CONTRAINDICATIONS: Leucovorin is improper therapy for pernicious anemia and other megaloblastic anemias secondary to the lack of vitamin B_{12} A hematologic remission may occur while neurologic manifestations remain progressive

WARNINGS: In the treatment of accidental overdosage of folic acid antagonists, leucovorin should be administered as promptly as possible As the time interval between antifolate administration (eg, methotrexate) and leucovorin rescue increases, leucovorin's effectiveness in counteracting hematologic toxicity diminishes

PRECAUTIONS: *General* Following chemotherapy with folic acid antagonists, parenteral administration of leucovorin is preferable to oral dosing if there is a possibility that the patient may vomit and not absorb the leucovorin In the presence of pernicious anemia, a hematologic remission may occur while neurologic manifestations remain progressive Leucovorin has no effect on other toxicities of methotrexate, such as the nephrotoxicity resulting from drug precipitation in the kidney

Drug Interactions Folic acid in large amounts may counteract the antiepileptic effect of phenobarbital, phenytoin, and primidone and increase the frequency of seizures in susceptible children

Pregnancy Teratogenic Effects Pregnancy Category C Animal reproduction studies have not been conducted with leucovorin It is also not known whether leucovorin can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity Leucovorin should be given to a pregnant woman only if clearly needed

Nursing Mothers It is not known whether this drug is excreted in human milk Because many drugs are excreted in human milk, caution should be exercised when leucovorin is administered to a nursing mother

Pediatric Use (See Drug Interactions)

ADVERSE REACTIONS: Allergic sensitization has been reported following both oral and parenteral administration of folic acid

OVERDOSAGE: Excessive amounts of leucovorin may nullify the chemotherapeutic effect of folic acid antagonists

DOSAGE AND ADMINISTRATION: Leucovorin is a specific antidote for the hematopoietic toxicity of methotrexate and other strong inhibitors of the enzyme dihydrofolate reductase Leucovorin rescue must begin within 24 hours of antifolate administration A conventional leucovorin rescue dosage schedule is 10 mg/m^2 orally or parenterally followed by 10 mg/m^2 orally every six hours for 72 hours If, however, at 24 hours following methotrexate administration the serum creatinine is 50% or greater than the premethotrexate serum creatinine, the leucovorin dosage should be immediately increased to 100 mg/m^2 every three hours until the serum methotrexate level is below 5×10^{-8}M[7,8] The recommended dosage of leucovorin to counteract hematologic toxicity from folic acid antagonists with less affinity for mammalian dihydrofolate reductase than methotrexate (ie, trimethoprim, pyrimethamine) is substantially less and 5 to 15 mg of leucovorin per day has been recommended by some investigators[9,10,11]

REFERENCES:

1 Nixon PF and Bertino JR Effective Absorption and Utilization of Oral Formyltetrahydrofolate in Man *N Engl J Med 286*(4) 175-179, Jan 1972
2. Ratanasthien K, Blair JA Leeming RJ, Cooke WT and Melikian V Folates in Human Serum *J Clin Pathol 27* 875-879, 1974
3 Mehta BM, Gisolfi AL, Hutchison DJ, Nirenberg A, Kellick MG and Rosen G Serum Distribution of Citrovorum Factor and 5-methyltetrahydrofolate Following Oral and I M Administration of Calcium Leucovorin in Normal Adults *Cancer Treat Rep* 62(3) 345-350, Mar 1978
4 Data on file, Clinical Research, Lederle Laboratories, Pearl River, NY 10965
5 Whitehead VM, Pratt R, Viallet A and Cooper BA Intestinal Conversion of Folinic Acid to 5-methyltetrahydrofolate in Man *Br J Haematol 22*:63-72, 1972
6 Herbert V Aseptic Addition for *Lactobacillus casei* Assay of Folate Activity in Human Serum *J Clin Path* 19 12-16, 1966
7 Bleyer WA The Clinical Pharmacology of Methotrexate *Cancer 41*(1) 36-51, 1978
8 Frei E, Blum RH, Pitman SW, *et al* High Dose Methotrexate with Leucovorin Rescue Rationale and Spectrum of Antitumor Activity *Am J Med* 68:370-376, 1980
9 Golde DW, Bersch N, Quan SG Trimethoprim and Sulphamethoxazole Inhibition of Haematopoiesis *in Vitro Br J Haematol, 40*(3) 363-367, 1978
10 Steinberg SE, Campbell CL, Rabinovitch PS, *et al* The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells *Blood*, 55(3) 501-504, 1980
11 Mahmoud AAF and Warren KS Algorithms in the Diagnosis and Management of Exotic Disease XX Toxoplasmosis *J Infect Dis 135*(3) 493-496, 1977

Rev 2/86

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Twenty years ago, leukemia* killed enough people to fill the Houston Astrodome.

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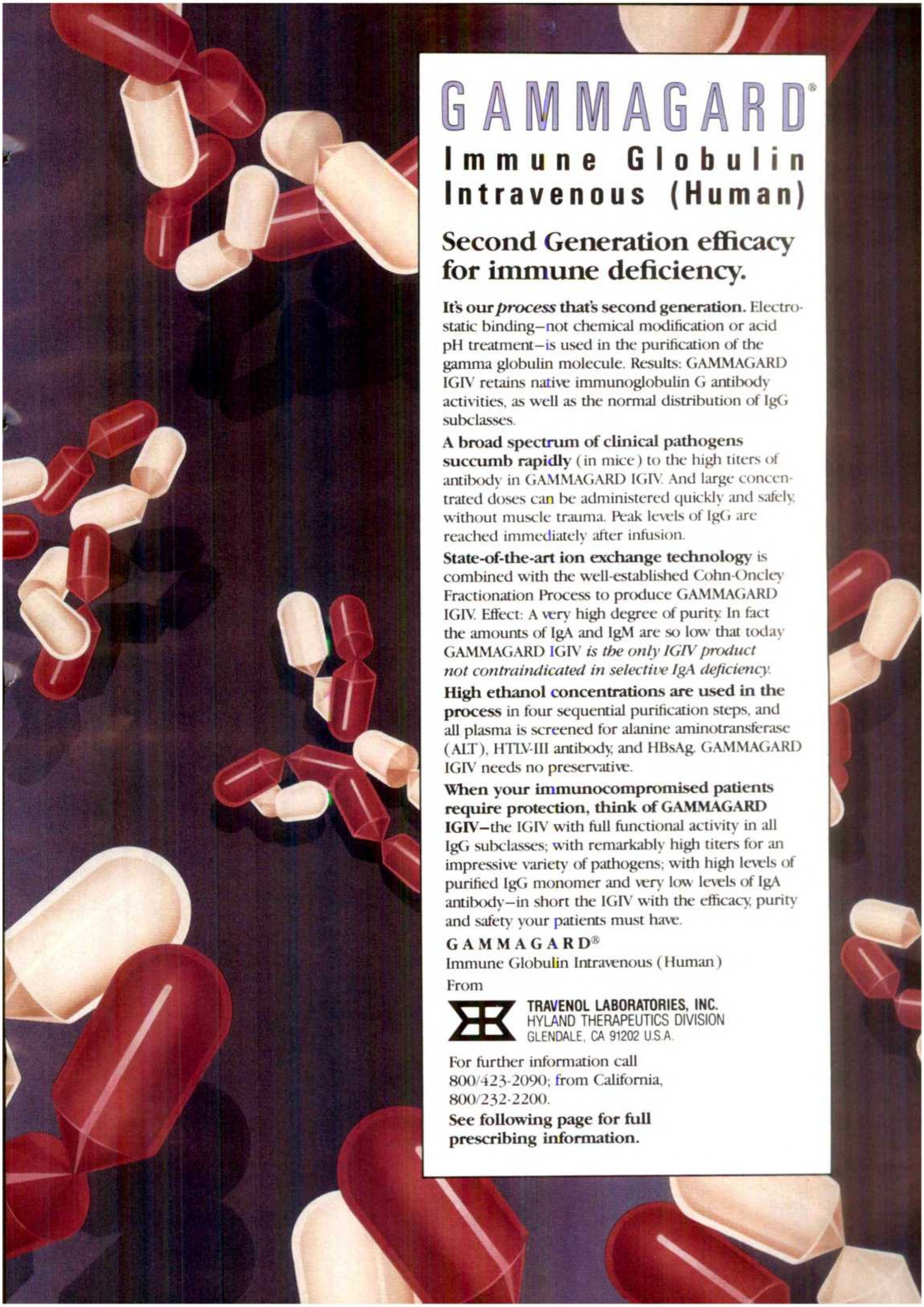

GAMMAGARD®

Immune Globulin Intravenous (Human)

Second Generation efficacy for immune deficiency.

It's our *process* that's second generation. Electrostatic binding—not chemical modification or acid pH treatment—is used in the purification of the gamma globulin molecule. Results: GAMMAGARD IGIV retains native immunoglobulin G antibody activities, as well as the normal distribution of IgG subclasses.

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High ethanol concentrations are used in the process in four sequential purification steps, and all plasma is screened for alanine aminotransferase (ALT), HTLV-III antibody, and HBsAg. GAMMAGARD IGIV needs no preservative.

When your immunocompromised patients require protection, think of GAMMAGARD IGIV—the IGIV with full functional activity in all IgG subclasses; with remarkably high titers for an impressive variety of pathogens; with high levels of purified IgG monomer and very low levels of IgA antibody—in short the IGIV with the efficacy, purity and safety your patients must have.

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For further information call
800/423-2090; from California,
800/232-2200.

See following page for full prescribing information.

DIRECTION INSERT

Immune Globulin Intravenous (Human) GAMMAGARD®

DESCRIPTION

Immune Globulin Intravenous (Human), GAMMAGARD® is a sterile, dried, highly purified preparation of immunoglobulin which is derived from the cold ethanol fractionation process and is further purified using ultrafiltration and ion exchange adsorption. When reconstituted with the appropriate volume of diluent, this preparation contains approximately 50 mg of protein per mL, of which at least 90% is gamma globulin. The reconstituted product contains approximately 1% sodium chloride, not more than 20 mg/mL glucose, not more than 0.2 g/dL PEG, and 0.3M glycine as a stabilizing agent. It has a pH of 6.8±0.4.

The manufacturing process for Immune Globulin Intravenous (Human), GAMMAGARD, isolates gamma globulin without additional chemical or enzymatic modification and the Fc portion is maintained intact. Immune Globulin Intravenous (Human), GAMMAGARD contains all the immunoglobulin G antibody activities which are present in the donor population. On the average the distribution of IgG subclasses present in this product is the same as is present in normal plasma.[1] Immune Globulin Intravenous (Human), GAMMAGARD contains only trace amounts of IgM and IgA.

Immune Globulin Intravenous (Human), GAMMAGARD, contains no preservative.

This product has been prepared from large pools of human plasma which was taken only from donors found to have normal levels of alanine aminotransferase (ALT). Each unit of plasma used in the manufacture of this product has been found to be nonreactive for HBsAg and HTLV-III Antibody by FDA approved tests.

CLINICAL PHARMACOLOGY

Immune Globulin Intravenous (Human), GAMMAGARD contains a broad spectrum of IgG antibodies against bacterial and viral agents that are capable of opsonization and neutralization of microbes and toxins.

Peak levels of IgG are reached immediately after infusion of Immune Globulin Intravenous (Human), GAMMAGARD. It has been shown that IgG is distributed relatively rapidly between plasma and extravascular fluid until approximately half of the total body pool is partitioned in the extravascular space. A rapid initial drop in serum level is, therefore, to be expected.[2]

As a class, IgG survives longer *in vivo* than other serum proteins.[2,3] Studies show that the half-life of Immune Globulin Intravenous (Human), GAMMAGARD is approximately 24 days. These findings are consistent with reports of a 21 to 25 day half-life for IgG.[2,3,4] The half-life of IgG can vary considerably from person to person, however. In particular, high concentrations of IgG and hypermetabolism associated with fever and infection have been seen to coincide with a shortened half-life of IgG.[2,3,4,5]

INDICATIONS AND USAGE

Antibody Deficiency

Immune Globulin Intravenous (Human), GAMMAGARD is efficacious in the treatment of primary immunodeficient states in which severe impairment of antibody forming capacity has been shown, such as: congenital agammaglobulinemias, common variable immunodeficiency, Wiskott-Aldrich syndrome, and severe combined immunodeficiencies.[4,5]

Immune Globulin Intravenous (Human), GAMMAGARD is especially useful when high levels or rapid elevation of circulating gamma globulins are desired or when intramuscular injections are contraindicated.

CONTRAINDICATIONS

None known.

WARNINGS

Immune Globulin Intravenous (Human), GAMMAGARD should only be administered intravenously. Other routes of administration have not been evaluated.

Immune Globulin Intravenous (Human), GAMMAGARD contains very low quantities of IgA (not more than 10 μg/mL) and although no instances of anaphylaxis associated with the use of this product have been observed during the clinical trials such reactions have been observed with other immunoglobulin products.[5,6] Immune Globulin Intravenous (Human), GAMMAGARD should be given with caution to patients with antibodies to IgA or selective IgA deficiencies.

PRECAUTIONS

Drug Interaction

Admixtures of Immune Globulin Intravenous (Human), GAMMAGARD with other drugs have not been evaluated. It is recommended that Immune Globulin Intravenous (Human), GAMMAGARD be administered separately from other drugs or medication which the patient may be receiving.

Pregnancy Category C

Animal reproduction studies have not been conducted with Immune Globulin Intravenous (Human), GAMMAGARD. It is also not known whether Immune Globulin Intravenous (Human), GAMMAGARD can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Immune Globulin Intravenous (Human), GAMMAGARD should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

The incidence of untoward reactions to Immune Globulin Intravenous (Human), GAMMAGARD is low, although various minor reactions, such as headache, fatigue, chills, backache, lightheadedness, fever and nausea were occasionally observed during our clinical trials. The incidence of these reactions during the clinical trials was less than 6%. Slowing or stopping the infusion usually allows the symptoms to disappear promptly.

Immediate anaphylactic and hypersensitivity reactions due to previous sensitization, although they have not been observed during the clinical trials are a possibility. Epinephrine should be available for treatment of any acute anaphylactoid reaction. (See WARNINGS).

DOSAGE AND ADMINISTRATION

Antibody Deficiency

For patients with primary immunodeficiencies, monthly doses of at least 100 mg/kg are recommended. Initially, patients may receive 200-400 mg/kg. As there are significant differences in the half-life of IgG among patients with primary immunodeficiencies, the frequency and amount of immunoglobulin therapy may vary from patient to patient. The proper amount can be determined by monitoring serum IgG levels before each dose, to insure that th do not drop below 500 mg/dL.

Rate of Administration

It is recommended that initially a rate of 0.5 mL/ per Hr be used. If infusion at this rate causes t patient no distress, the administration rate may gradually increased but should not exceed 4 mL/ per Hr.

A rate of administration which is too rapid m cause flushing and changes in pulse rate and blo pressure. Slowing or stopping the infusion usua allows the symptoms to disappear promptly.

Administration

Immune Globulin Intravenous (Humar GAMMAGARD should be administered as soon aft reconstitution as possible. Administration should b gin not more than 2 hours after reconstitution.

The reconstituted material should be at roo temperature during administration.

Parenteral drug products should be inspect visually for particulate matter and discoloration pri to administration, whenever solution and contain permit.

Follow directions for use printed on the administ tion set container. If a Hyland set is not used, ma sure the administration set contains an adequate filt

How Supplied

Immune Globulin Intravenous (Huma GAMMAGARD is supplied in either 2.5 g or 5.0 g sin use vials. Each vial of Immune Globulin Intraveno (Human), GAMMAGARD is furnished with a suitab volume of Sterile Water for Injection, USP, a trans device and an administration set which contains integral airway and a 15 micron filter.

Storage

Immune Globulin Intravenous (Huma GAMMAGARD should be stored under ordin refrigeration, (2 to 8 °C, 36 to 46 °F). Freezing sho be avoided to prevent the diluent bottle from breakir

REFERENCES

1. Unpublished data in files of Travenol Laborator Inc.
2. Waldman TA, Storber W: Metabolism of immur globulins. *Prog Allergy 13*: 1-110, 1969.
3. Morell A, Riesen W: Structure, function and ca bolism of immunoglobulins in *Immunohemothera* Nydegger UE (ed), London, Academic Press, 19 pp 17-26.
4. Stiehm ER: Standard and special human immu serum globulins as therapeutic agents. *Pediatr* 63:301-319, 1979.
5. Buckley RH: Immunoglobulin replacement thera Indications and contraindications for use and varia IgG levels achieved in *Immunoglobulins: Charact istics and Use of Intravenous Preparations.* Alv BM, Finlayson JS (eds), Washington, D.C., U.S. partment of Health and Human Services, 1979, pp
6. Burks AW, Sampson HA, Buckley RH: Anaphyla reactions following gammaglobulin administratior patients with hypogammaglobulinemia: Detection IgE antibodies to IgA. Submitted for publicati

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(Continued on following page)

(Continued from preceding page)

PREPARING THE MANUSCRIPT

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The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which work was done; (4) acknowledgments for research support; (5) name and address of the author to whom communications regarding the manuscript should be directed.

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Tables and illustrations must be cited in order in the text using arabic numerals. All line drawings should be submitted as clear, glossy, black and white photographs. Hand-drawn or hand-lettered illustrations are unacceptable. Legible copies may be used only with the duplicate manuscripts. Photomicrographs and other photographic illustrations must be submitted in triplicate; photocopies of photographs and line drawings are not acceptable. The name of the first author, figure number, and designation of the top of the illustration should be marked on the back of each illustration with a soft lead pencil. Authors should avoid mounting illustrations on boards unless mounting is necessary to insure proper placement. Legends for illustrations should be typewritten, double spaced, on a separate sheet, and included at the end of the manuscript. A legend must accompany each illustration. Contributors will pay all charges involved in processing and printing of color photographs.

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EXAMPLES OF REFERENCES

Journal article, one or more authors:

1. Golomb HM, Vardiman J, Sweet DL, Simon D, Variakojis D: Hairy cell leukemia: Evidence for the existence of a spectrum of functional capabilities. Br J Haematol 38:161, 1978

Journal article, in press:

2. O'Malley JE, Eisenberg L: The hyperkinetic syndrome. Semin Psychiatry (in press)

Complete book:

3. Lillie RD: Histopathologic Technic and Practical Histochemistry (ed 3). New York, Blakiston, 1965, p 39

Chapter of book:

4. Moore G, Minowada J: Human hemopoietic cell lines: A progress report, in Farnes P (ed): Hemic Cells In Vitro, vol 4. Baltimore, Williams & Wilkins, 1969, p 100

Chapter of book that is part of published meeting:

5. Natvig JB, Kunkel HG, Gedde-Dahl T Jr: Genetic studies of the heavy chain sub-groups of G globulin, in Killander J (ed): Gamma Globulins, Proceedings of the Third Nobel Symposium. New York, Wiley, 1967

Chapter of book that is part of unpublished meeting:

6. Polliak A: A morphologic study of the lymphoproliferative lesions induced by excess vitamin A. First Meeting, European Division, International Society of Hematology, Milan, 1971, p 181

Abstract:

7. Curnutte JT, Karnovsky ML, Babior BM: Manganese-dependent NADPH oxidation by a particulate preparation from guinea pig granulocytes: An alternative interpretation. Clin Res 23:271A, 1975 (abstr)

Letter to the Editor:

8. Seeler RA: Sickle cell anemia monthly variations. Blood 47:879, 1976 (letter)

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[1] Rubin, L. A., C. C. Kurman, M. E. Fritz, W. E. Biddison, B. Boutin, R. Yarchoan, and D. L. Nelson. 1985. Soluble interleukin 2 receptors are released from activated human lymphoid cells in vitro. **The Journal of Immunology** 135:3172.

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REVIEW

Thrombolytic Therapy in the Eighties

By Marc Verstraete and Désiré Collen

WHEN A FIBRIN thrombus impedes blood flow in an artery or a vein, subsequent events partly depend on the fate of the thrombus, which may fragment and embolize, undergo organization, or lyse. There is deceivingly sketchy information on the first two processes. However, knowledge of the intricate system of physiological fibrinolysis is rapidly growing and, as a result, a new generation of thrombolytic drugs is being developed.

THE MAIN COMPONENTS OF THE FIBRINOLYTIC SYSTEM

The human fibrinolytic system comprises a proenzyme, plasminogen, which can be activated to the active enzyme, plasmin, by several types of plasminogen activators (Fig 1). Inhibition of the fibrinolytic system may occur at the level of plasmin or at the level of the plasminogen activation. Although plasmin is known to possess a multiplicity of functions,[1-14] only its role in fibrinolysis and thrombolysis is dealt with in this overview.

Plasminogen. Human plasminogen is a single-chain glycoprotein with a mol wt of ~90 kd; its concentration in plasma is 20 mg per deciliter (2.2 μmol/L). The molecule consists of 790 amino acids; it contains 24 disulfide bridges and five homologous triple-loop structures or "kringles" of ~10 kd each.[15] These kringles are disulfide structures containing the lysine-binding sites which mediate its interaction with fibrin,[16] with α_2-antiplasmin,[17] and with histidine-rich glycoprotein,[18] but also with the synthetic amino acids such as 6-aminohexanoic acid (EACA) and tranexamic acid.[19]

Native plasminogen has NH_2-terminal glutamic acid ("Glu-plasminogen") and can occur in two major variants (I and II) that differ in carbohydrate content[20,21]; the two molecular forms appear to be synthesized in liver parenchymal cells.[22] Glu-plasminogen can easily be converted by limited plasmic digestion to modified forms with NH_2-terminal lysine, valine, or methionine,[23] commonly designated "Lys-plasminogen." During this degradation process, a 8-kd peptide is removed from the NH_2-terminal end of the Glu-plasminogen. The conversion of single-chain Lys-plasminogen to plasmin is due to cleavage of a single Arg 560-Val 561 bond.[24] The resulting two-chain plasmin molecule is composed of a heavy chain (A chain) originating from the NH_2-terminal part of plasminogen, and a light chain (B chain) constituting the COOH-terminal part.[25] The B chain was found to contain the active catalytic site similar to that of trypsin.[15]

α_2-Antiplasmin. This inhibitor is a single-chain glycoprotein with a mol wt of 67 kd[26,27] and its concentration in plasma is 6.9 mg per deciliter (1 μmol/L). α_2-antiplasmin belongs to the same protein family as antithrombin III, α_1-antitrypsin and ovalbumin.[28] In purified systems[26,27] and in plasma[29,30] α_2-antiplasmin forms a 1:1 stoichiometric complex with plasmin by strong interaction between the light (B)-chain of plasmin and the inhibitor. The complex is devoid of protease or esterase activity. The time course of the inhibition of human plasmin by α_2-antiplasmin is compatible with a kinetic model composed of two successive reactions: a very fast, reversible, second-order reaction followed by a slower, irreversible, first-order reaction.[31,32] The former is among the fastest protein–protein reactions so far described. In spite of the presence of other plasmin inhibitors in plasma (eg, α_2-macroglobulin) the inhibition of this enzyme is solely and instantaneously carried out by α_2-antiplasmin. Plasmin molecules which have a synthetic substrate bound to their active site or 6-aminohexanoic acid bound to their lysine-binding site(s)[32] either do not react or react only very slowly with α_2-antiplasmin. The first step of the process is thus clearly dependent on the presence of a free lysine-binding site and active site in the plasmin molecule.

Tissue-type plasminogen activator. Tissue-type plasminogen activator (t-PA) is a serine protease with a mol wt of ~70 kd. t-PA obtained from Bowes melanoma cells appears to exist in two variants, with an apparent mol wt difference of 3 kd.[33,34] The concentration of t-PA in human plasma is ~5 ng per milliliter (~0.1 nmol/L). Human t-PA consists of one polypeptide chain containing 527 amino acids exhibiting serine as the NH_2-terminal amino acid.[35] Upon limited plasmic action, the molecule is converted to a two-chain activator linked by disulfide bonds.[36-38] This occurs by cleavage of the Arg 275-Ile 276 peptide bond, yielding a heavy chain (36 kd) derived from the NH_2-terminal part of the molecule and a light chain (32 kd) comprising the

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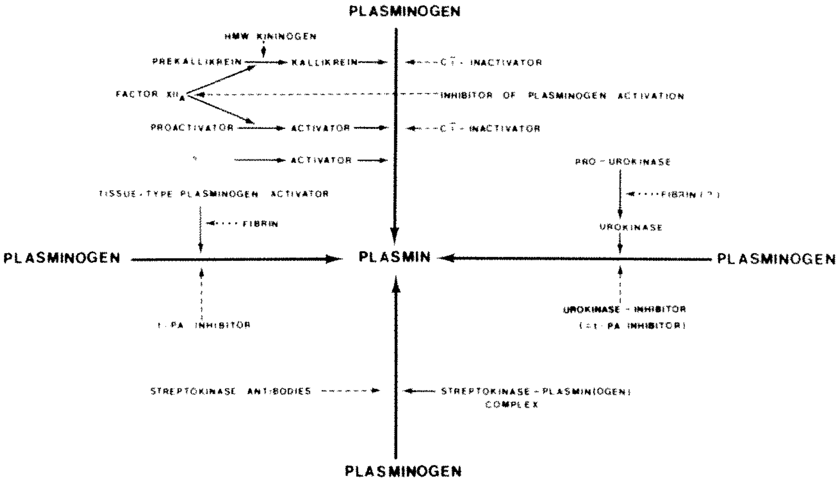

Fig 1. Schematic representation of activation pathways of plasminogen to plasmin (—→), and site of action of inhibitors (---→) and of cofactors (····→). Pro-urokinase is synonym of single-chain urokinase-type plasminogen activator.

COOH-terminal region. The heavy chain contains two regions that share a high degree of homology with the five kringles of plasminogen and with the single kringle in urokinase (Fig 2). The molecule contains an NH_2-terminal region which is homologous with the finger domains responsible for the fibrin-affinity of fibronectin[39] and another sequence having a certain analogy with human epidermal growth factor. The catalytic site is located in the light or B-chain of t-PA; it contains the three typical amino acids common to all trypsin-like serine proteases: histidine in position 325, aspartic acid in position 374, and serine in position 481, and is highly homologous to corresponding parts of trypsin, thrombin, plasmin, and elastase.[35,40] The one-chain and two-chain forms of t-PA have different amidolytic activities towards low mol wt substrates.[41] They have, however, virtually the same fibrinolytic activity in a purified system; the plasminogen-activating properties are also similar.[37,42] As explained more in detail in the next section, t-PA is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances the activation rate of plasminogen. This can be explained by an increased affinity of fibrin-bound t-PA for plasminogen, resulting in a ternary complex. This high affinity of t-PA for plasminogen in the presence of fibrin allows efficient activation on the fibrin clot without significant plasminogen conversion by t-PA in circulating plasma in animals.[36,42]

Several laboratories have obtained evidence for the existence of rapidly acting inhibitor(s) of t-PA at low concentrations in plasma of healthy individuals[43-46] or at higher levels in pathological plasma samples.[47-49] The complex formed between t-PA and its specific inhibitor has a mol wt of 110 kd and is formed very rapidly (second-order rate constant 10^7 mol/L) m^{-1} s^{-1}).[50]

Urokinase and single-chain urokinase-type plasminogen activator (pro-urokinase). Urokinase is a trypsin-like serine protease composed of two polypeptide chains (20 and 30 kd) connected by a single disulfide bridge. There are two molecular forms, designated S_1 (31 kd) and S_2 (54 kd), the lower mol wt form being a proteolytic degradation of the latter.[51] Urokinase is isolated from human urine or cultured human embryonic kidney cells.

Several groups have reported the isolation of an inactive single-chain precursor (pro-urokinase) of urokinase which, following limited digestion with plasmin, is converted to fully active two-chain urokinase.[52-58] Pro-urokinase is a true enzyme, as addition of a plasmin inhibitor (aprotinin or α_2-antiplasmin) abolishes the conversion of pro-urokinase to urokinase but not the activation of plasminogen to plasmin in purified systems.[59] Consequently, the designation single-chain urokinase-type plasminogen activator (scu-PA) for this single-chain precursor form of urokinase was proposed by the International Committee on Thrombosis and Haemostasis instead of pro-urokinase (annual meeting, San Diego, 1985). Scu-PA is, however, inactive in human plasma due to the presence of a competitive inhibitor[60] (Fig 3). If fibrin is present, this inhibition is abolished and plasminogen activation occurs, resulting in formation of fibrin-associated plasmin.[61] The apparent fibrin-specificity of scu-PA thus seems to be due to the fact that fibrin neutralizes this competitive inhibitor, and not to a fibrin-enhanced activation of plasminogen as is the case for t-PA.

The gene coding for urokinase has recently been cloned and expressed in *E coli*.[62] However, if proteolytic degradation is carefully avoided during purification, scu-PA may be obtained from the same expression system.[63] Natural and recombinant scu-PA can induce more clot-selective thrombolysis than natural or recombinant urokinase in experimental thrombotic models.[53,64,66]

Inactivation by plasma protease inhibitors does not seem to play a significant role in the inhibition of urokinase in vivo,[65] although recently a new fast-acting inhibitor of urokinase has been detected in low concentrations in the blood.[67]

Streptokinase. Streptokinase is a nonenzyme protein with a mol wt of 47 kd, produced by Lancefield group C strains of β-hemolytic streptococci. Streptokinase initially forms a 1:1 stoichiometric complex with plasminogen which then undergoes a transition, allowing formation of a complex that possesses an active site in the modified plasminogen moiety. This complex enzymatically converts plasminogen to plasmin.[68]

Human plasma contains antibodies directed against streptokinase, which most probably result from previous infec-

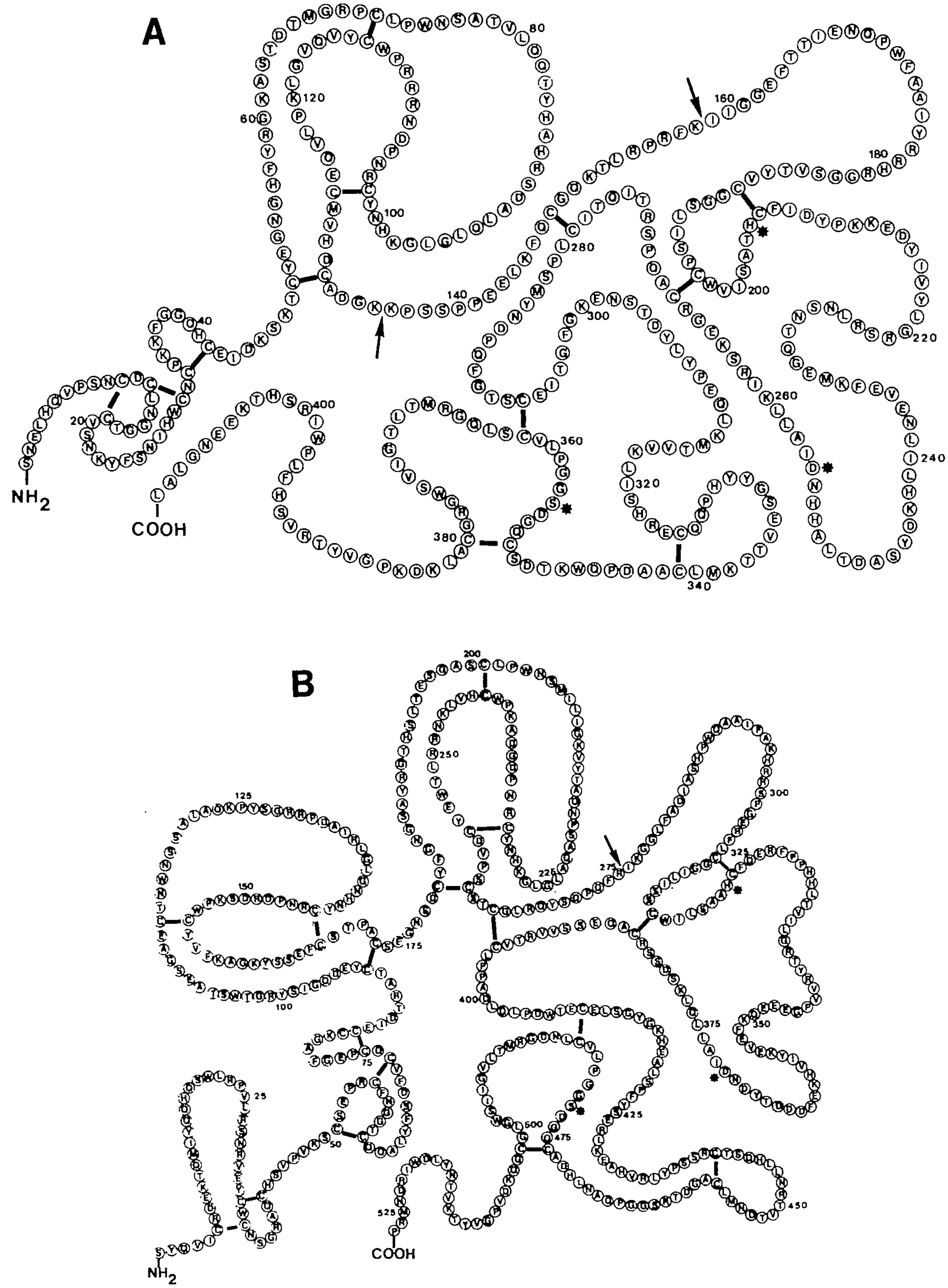

Fig 2. Structure of single-chain urinary-type plasminogen activator (scu-PA) (A), tissue-type plasminogen activator (t-PA) (B) and plasminogen (C).

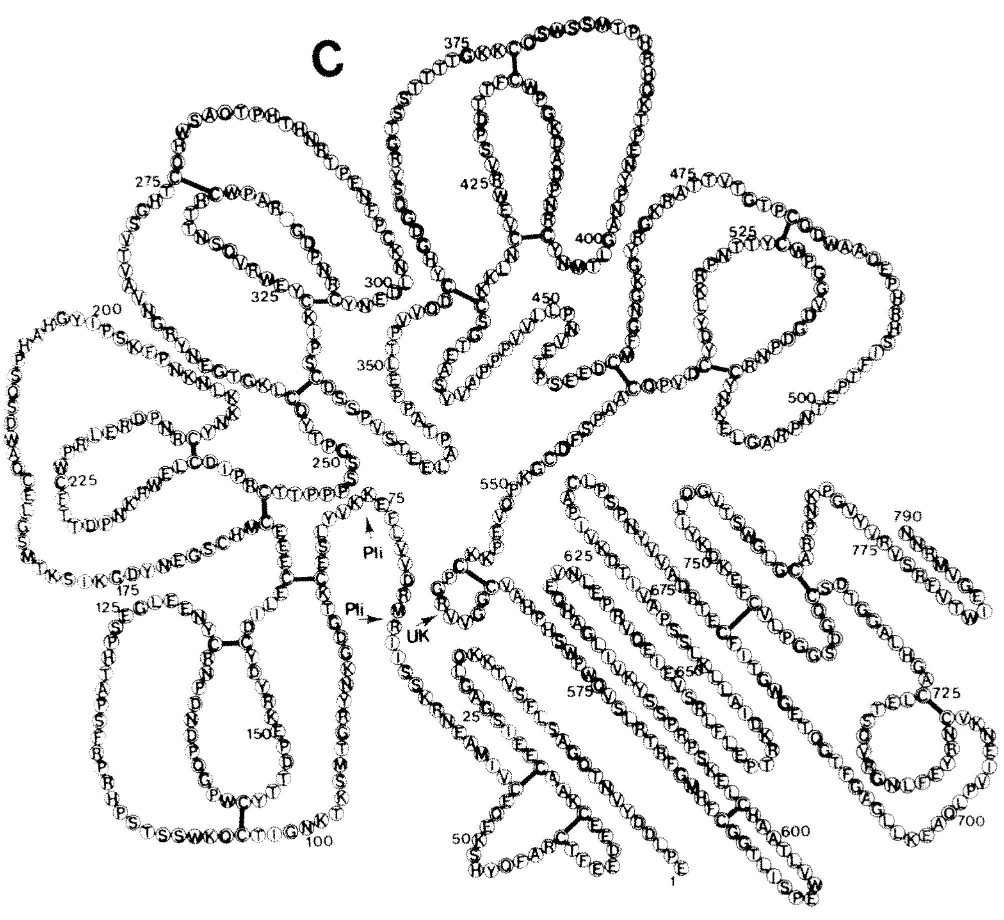

Fig 2. (Cont'd)

tions with β-hemolytic streptococci. Moreover, streptokinase is immunogenic in humans. Because streptokinase reacts with antibodies and is thereby rendered biochemically inert, sufficient amounts of streptokinase must be infused to neutralize the antibodies before fibrinolytic activation is obtained.[69]

REGULATION OF THE FIBRINOLYTIC SYSTEM IN HUMANS

Different, apparently divergent concepts have been developed to explain why plasmin, formed in the vicinity of thrombi, is not inactivated by circulating antiplasmins. Alkjaersig and colleagues[70] were the first to propose that plasminogen is adsorbed to fibrin when the latter is formed. The in situ activation of fibrin-bound plasminogen was considered to be achieved by activators, diffusing from the plasma into the thrombus, generating plasmin in an environment shielded from circulating antiplasmins. More particularly, Lys-plasminogen, which is generated from the native Glu-plasminogen, was shown to have a higher affinity for fibrin in a purified system[71] and in plasma.[72] The lysine-binding site(s) of plasminogen mediate its binding to fibrin[17] and are also responsible for the plasmin-α_2-antiplasmin interaction. It thus appears that by competing with fibrin for the same lysine-binding sites in plasminogen, α_2-antiplasmin inhibits the binding of plasminogen to fibrin. According to Aoki and Sakata, α_2-antiplasmin cross-links with fibrin but still has a plasminogen-binding capacity, resulting in an indirect binding of plasminogen to fibrin.[73] Previous conflicting reports concerning the uptake of plasminogen by thrombi may have been due to a lack of distinction between the Glu- and Lys-forms of plasminogen as well as to differences in techniques and substrates used.[74-76]

Another school of thought held that plasminogen activators were primarily adsorbed onto fibrin, and that the rate of fibrinolysis was dependent on the level of circulating plasminogen.[77-83] Chesterman et al[77] demonstrated a relative affinity of streptokinase-plasminogen activator for fibrin. Subsequently, a specific affinity of plasma plasminogen activator for fibrin was demonstrated.[78-81] Purified tissue-type plasminogen activator (t-PA) binds specifically to

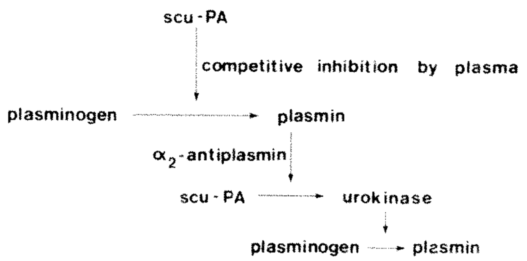

Fig 3. Hypothetical mechanism for the activation of plasminogen by single-chain urinary-type plasminogen activator (scu-PA).

fibrin[82-85] or to CNBr-digested fibrinogen fragments.[45] Quantitative data have been obtained on t-PA binding to fibrin and on the role of fibrin in the activation of plasminogen by t-PA.[86-88] It was shown that t-PA has a weak affinity for plasminogen ($K_m = 65\ \mu mol/L$) in the absence of either solid-phase fibrin or a CNBr-digested fibrinogen, but a much higher affinity when fibrin is present ($K_m = 0.16\ \mu mol/L$). No difference was found in these kinetic parameters for either the t-PA of melanoma origin[36] or obtained by recombinant DNA technology.[35] The structures involved in the binding of t-PA to fibrin are not clearly defined. The NH_2-terminal region of t-PA, which is homologous to the finger-region of fibronectin, has been implicated,[39] but recent observations seem to be at variance with this hypothesis.[89]

It had previously been shown that plasmin is extremely rapidly inactivated by α_2-antiplasmin, but plasmin with occupied lysine-binding sites is inactivated 50 times slower by α_2-antiplasmin. Reversible blocking of the active site of plasmin with substrate also markedly reduces the rate of inactivation by α_2-antiplasmin. A molecular model was proposed for the regulation of fibrinolysis in vivo that retains elements of each of the former concepts[17,32,90] (Fig 4). As noted above, t-PA has a weak affinity for plasminogen in the absence of fibrin but a much higher affinity when fibrin is present. Moreover, the binary complex formed by t-PA and fibrin has a high affinity for plasminogen ($K_m = 0.14\ \mu mol/L$). This increased affinity appears to be the result of a "surface assembly" of t-PA and plasminogen on the fibrin surface. Fibrin essentially increases the local plasminogen concentration, creating an additional interaction between t-PA and its substrate through a cyclic fibrin bridge, resulting in a low Michaelis constant for the activation of plasminogen by t-PA.[86] Plasmin formed on the fibrin surface has both its lysine-binding sites and active site occupied and is thus only slowly inactivated by α_2-antiplasmin [half-life (t½) of ~10 s]. Liberated plasmin, however, is very quickly inactivated by α_2-antiplasmin (t½ of ~100 ms). Effective thrombus dissolution in vivo thus requires a continuous replacement at the fibrin surface of consumed plasmin by new plasminogen molecules.

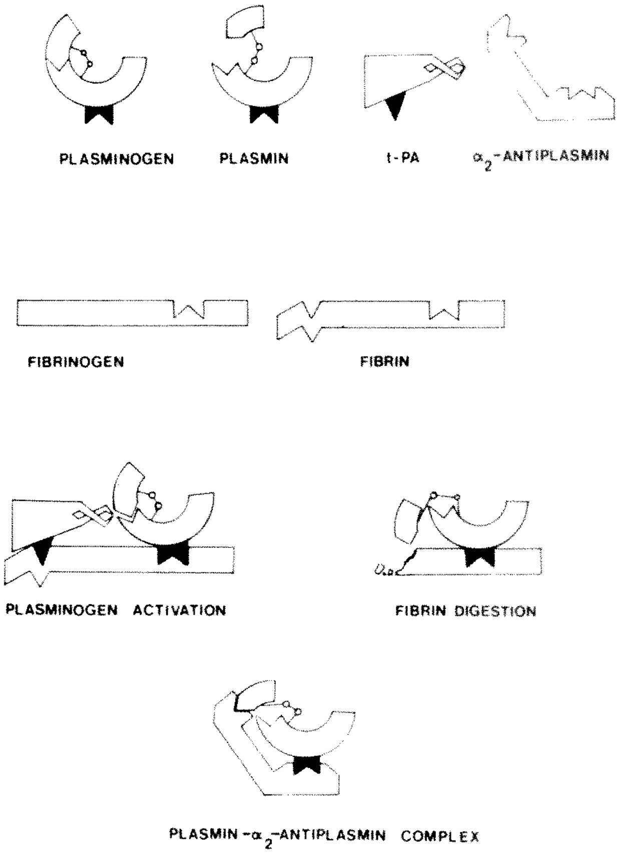

Fig 4. **Schematic visualization of the molecular interactions regulating fibrinolysis. On the fibrin surface, plasminogen is efficiently converted to the proteolytic enzyme plasmin by bound plasminogen activator. The plasmin generated is partially protected from inactivation by α_2-antiplasmin, whereas free plasmin in the blood is very rapidly inactivated. The lysine-binding sites of plasminogen (■) are important for the interaction between plasmin(ogen) and fibrin and between plasmin and α_2-antiplasmin; active site of plasmin (▼).**

MAJOR CLINICAL ACHIEVEMENTS WITH STREPTOKINASE AND UROKINASE

Uncertainty about the optimal dose and, above all, fear of bleeding and other complications result in an underuse of streptokinase and urokinase, as was apparent at an NIH Consensus Development Conference.[91] It is mainly the risk versus a too uncertain benefit assessment that is hampering clinicians in making a more liberal use of presently available thrombolytic agents, particularly in the postoperative period, after injury and stroke, and in patients who have undergone invasive procedures. The observation that there appears to be a poor correlation between the laboratory parameters of systemic fibrinolysis and the incidence of bleeding or the degree of thrombus resolution is also vexing.[92]

A well-studied indication for thrombolytic therapy is major pulmonary embolism. Timely administration of streptokinase and urokinase results in a greater resolution of massive pulmonary emboli and greater improvement of the hemodynamic cardiopulmonary measurements as compared with heparin treatment alone, as was shown in the trials with urokinase and streptokinase sponsored by the National Institutes of Health (NIH).[93-95] The number of patients in these trials was, however, too small to demonstrate a significant reduction in mortality. Moreover, only 10% of the patients included in the NIH-sponsored trials were in shock; comparative trials limited to patients with pulmonary embolism in shock may reveal a lower mortality rate in patients treated with thrombolytic agents without requiring an exceedingly large number of patients in the trial. A long-term benefit of thrombolytic treatment in major pulmonary embolism is that complete resolution of thromboemboli, also from the pulmonary microcirculation, has reduced the incidence of chronic pulmonary hypertension.[96]

A multicenter trial was set up in France to compare a 12-hour treatment with 4,400 CTA U urokinase per kilogram per hour and a 24-hour treatment with half this hourly dose in patients with recent pulmonary embolism.[97] The angiographic results and bleeding complications were not significantly different between the two regimens. However, either treatment tested would be too short to dissolve concomitant deep vein thrombi effectively, which requires 4 to 5 days of intense thrombolysis.[98–99] The best prevention of recurrent pulmonary embolism is indeed complete phlebographic clearance of deep vein thrombosis.

In the presence of a massive, life-threatening pulmonary embolism in a patient with cardiovascular collapse, the physician in charge must make a rapid decision as to whether to begin thrombolytic treatment or to mobilize heart-lung surgeons to perform an urgent embolectomy. No trial has been conducted in this dramatic setting; the results of such a trial could guide physicians in decision making.

At least seven randomized and controlled studies with streptokinase in patients with deep vein thrombosis have been conducted in which the efficacy was based upon interpretation of phlebograms by uninformed radiologists; these findings were critically reviewed and well expounded by Marder.[92] No individual trial had a sample of sufficient size to determine both efficacy and safety with adequate power. An aggregate of six randomized trials in which phlebography was used to assess thrombolytic treatment revealed that thrombolysis was achieved 3.7 times more often in streptokinase-treated patients than in heparin-treated patients ($P < 0.001$), but bleeding was 2.9 times greater in the first group ($P < 0.04$).[100] Whether the increased rate of resolution of deep vein thrombi with thrombolytic agents as compared with heparin will preserve valvular function and reduce long-term chronic venous insufficiency has yet to be proven.[101]

Attempts to recanalize the infarct-related coronary artery in patients with acute myocardial infarction, either with intracoronary or systemic administration of streptokinase or urokinase, resulted in a reperfusion of 75% and 45% of the patients, respectively.[102] The treatment is more likely to reopen the coronary artery when begun relatively early (less than four hours after onset of symptoms). It is a reasonable assumption, based on earlier and later trials with intravenous (IV) and intracoronary fibrinolytic treatment,[103–105] that timely reopening of a thrombosed coronary artery will result in salvage of contractile myocardium, decrease early and late mortality, and improve the quality of life in patients with acute myocardial infarction. This hypothesis is now under investigation in several multicenter trials, conducted both in Europe and in the United States.

THE FEAR OF BLEEDING HAS CONSIDERABLY HAMPERED THROMBOLYTIC TREATMENT WITH SYSTEMICALLY ADMINISTERED STREPTOKINASE AND UROKINASE

The comparatively high incidence of bleeding in the trials sponsored by the NIH in patients with pulmonary embolism (45% in those receiving urokinase or streptokinase treatment and 27% in those receiving heparin)[93–95] has impressed the medical community more than the faster resolution of the pulmonary emboli in the first group. Admittedly, bleeding was directly traceable to and occurred mainly at the immediate site of the multiple invasive procedures requested by the protocol. By avoiding nonessential invasive procedures and with proper attention to details, bleeding during thrombolytic treatment with streptokinase and urokinase is at present ~5%.[103,106–108] Nevertheless, a major concern is the ~1% to 3% incidence of cerebral hemorrhage, even when patients with severe hypertension, recent strokes, and advanced cerebrovascular diseases are excluded from thrombolytic treatment.[109] In a postmarketing surveillance program on streptokinase, 1% of the patients died directly due to this treatment.[110] The bleeding complications are the main cause of the lack of enthusiasm for the use of streptokinase and urokinase.

There appears to be a relationship between hemorrhagic complications and the duration of thrombolytic treatment,[111] despite the fact that fibrinogen levels rise progressively after 24 hours of continuous streptokinase administration.[112] To circumvent this drawback, intermittent infusions of streptokinase have been tried. When given at 24-hour intervals, a daily 30-minute infusion of 250,000 U of streptokinase resulted in a fibrinogen level oscillating between 20% and 50% of pretreatment concentration.[113] Variations with dosages and schedules of streptokinase administration indicated a dissatisfaction with the routine regimen. The application of coronary angiography in patients with acute myocardial infarction enabled a short-term, direct perfusion of the obstructed vessel with thrombolytic agents as well as direct observation of the desired thrombolytic effect.[114] A mean total dose of ~200,000 U of streptokinase is administered in the coronary artery over one hour to 90 minutes; hemorrhagic complications are generally confined to the site of angiocatheter insertion. Nevertheless, even this lower dose and shorter perfusion time are still associated with a fibrinogen decrease to 83% of the preinfusion value,[115,116] and bleeding requiring blood transfusion in 7% of the patients.[117,118] Spurred by the experience gained with intracoronary infusion of streptokinase, local perfusion of peripheral arterial occlusions accessible to catheter placement has resulted in a high success rate, a considerably shorter duration of thrombolytic treatment, and a low bleeding incidence at dosages not exceeding 5,000 IU streptokinase per hour.[119–122]

An additional problem with streptokinase, besides bleeding, is that most patients have significant antistreptokinase titers, as a result of previous streptococci infections. Thus, a loading dose of streptokinase must be given to saturate the antibody-binding capacity. The required initial dose can be calculated after in vitro titration; however, from a practical point of view, large doses of streptokinase are generally given to overcome circulating antibodies.[123] Some patients complain of lumbar pain during the rapid infusion of a high initial dose of streptokinase, and a temperature elevation >0.9 °C occurs in 25% of patients, even with highly purified preparations.[112] Minor, but occasionally major, allergic reactions to streptokinase are not uncommon and have been reported at a frequency of 1.7% to 18%.[124] Serum sickness with leucocytoclastic vasculitis is another rare complication attributed to delayed hypersensitivity reaction precipitated by exposure to the foreign protein streptokinase.[125,126] A transient rise in the serum transaminase (aspartate aminotransferase, alanine aminotransferase) levels has been reported during streptokinase treatment; its mechanism remains unclear.[127] Stoichiometric complexes of streptokinase with plasminogen are still antigenic and immunogenic in humans.[128]

As compared with streptokinase, urokinase has the advantage of being nonantigenic; its pyrogenicity is low and depends on the purity of the preparation. Urokinase also requires an initial high loading dose and, like streptokinase, possesses little affinity for fibrin[61,129] and therefore does not discriminate between circulating plasminogen and fibrin-bound plasminogen, which results in systemic activation of the fibrinolytic system. Urokinase is cleared from the circulation more rapidly than is streptokinase.[130]

Because t-PA is a circulating human protein, the likelihood of induction of allergic reactions is very low. Untoward reactions to t-PA have not been reported in the limited number of patients so far treated.

MOVING TOWARDS MORE SPECIFIC AND RAPID THROMBOLYSIS

The molecular model for the regulation of fibrinolysis described above has important implications for the development of novel thrombolytic agents. Indeed, the presently available thrombolytic agents streptokinase and urokinase have no specific affinity for fibrin and will therefore activate circulating and fibrin-bound plasminogen relatively indiscriminately. Any plasmin formed in the circulation will thus initially be neutralized rapidly by α_2-antiplasmin and be lost for thrombolysis. When the α_2-antiplasmin level is markedly reduced plasmin is less rapidly neutralized and can exert its proteolytic effect on several plasma proteins, among which the coagulation components fibrinogen and factors V and VIII are the most important. Excessive lowering of the concentration of these blood coagulation factors in plasma combined with the inhibitory effect of some fibrinogen degradation products on platelet aggregation and fibrin polymerization are held responsible for the potentially dangerous bleeding tendency.[131–133] However, the magnitude of systemic fibrinolysis and its laboratory derangements do not necessarily correlate with bleeding manifestations.[92,107,124] Thus, in one study it was shown that fibrinogen and plasminogen decreased to lower levels after streptokinase than after urokinase treatment, without apparent difference in the incidence of bleeding.[106] A plausible explanation would be that the activated systemic fibrinolytic system results in dissolution of hemostatic plugs.[92]

Fibrin-specific thrombolytic agents as tissue-type plasminogen activator and single-chain urokinase-type plasminogen activator may be expected to cause less bleeding complications resulting from a systemic fibrinogenolysis at doses that are therapeutically effective. However, a fibrin-specific agent is not expected to differentiate between the fibrin in the occluding thrombus and the fibrin around aggregated platelets in a hemostatic plug. Nonetheless, bleeding from fresh surgical wounds was not evident when t-PA was used in various animal models of thrombosis.[134–138]

Tissue-type plasminogen activator. Experience with recombinant t-PA, produced on a small scale for initial clinical evaluation (g11021) is limited to ~500 patients with acute myocardial infarction.[139–144] With the doses that were used, a drop in fibrinogen was noted to be about 50% of the preinfusion value. It was shown that systemic activation of the fibrinolytic system is dependent on the dose and infusion rate,[145] as could be anticipated from the kinetic parameters of the activation of plasminogen by t-PA. This fibrinogen decrease might be due in part to in vitro fibrinogen degradation. The fibrinogen level dropped (also by 11%) in the placebo-treated patients, indicating that coronary catheterization and hemodilution could also be partly responsible.[144] Hematoma at catheter site and prolonged bleeding at puncture sites combined, were half as frequent during t-PA treatment compared to streptokinase treatment in a comparative trial.[143] Further experience is required to substantiate whether less bleeding is encountered when using t-PA for therapeutic thrombolysis, compared with streptokinase or urokinase (Table 1).

In the two European trials with t-PA,[143,144] the observed increase in fibrinogen degradation products was less than anticipated from the reduction of circulating fibrinogen.[146] The fibrinogenolytic breakdown may be limited to the generation of the early degradation products fragments X and/or Y, which are slowly coagulable or incorporated in fibrin clots. This hypothesis is further supported by the less substantial decrease in fibrinogen during t-PA treatment when measured by the sodium sulphite precipitation method,[147]

Table 1. Main Characteristics of Presently Available Thrombolytic Agents

	Streptokinase	Urokinase	Acylated Streptokinase-Plasminogen	Recombinant Tissue-Type Plasminogen Activator	Single-Chain Urokinase-Type Plasminogen Activator
Systemic activation of the fibrinolytic system with fibrinogenolysis	+++	+++	++	+ or −	+ or −
Circulating antibodies	Yes	No	Yes	No	No
Circulating inhibitors	No	Yes	No	Yes	Yes
Antigenicity	Yes	No	Yes	No	No
Bleeding risk	Yes	Yes	Yes	Minor	Minor
Allergic reactions	Yes	No	Yes	No	No
Other side effects	Yes	No	Yes	No	No
Bolus or infusion	Infusion	Infusion	Bolus injection	Infusion	Infusion

Trade names for streptokinase—Kabikinase, Streptase; for urokinase—Abbokinase, Actosolv, Ukidan, and Persolv; for recombinant tissue type plasminogen activator—Activase; and for acylated streptokinase-plasminogen-APSAC, Iminase.

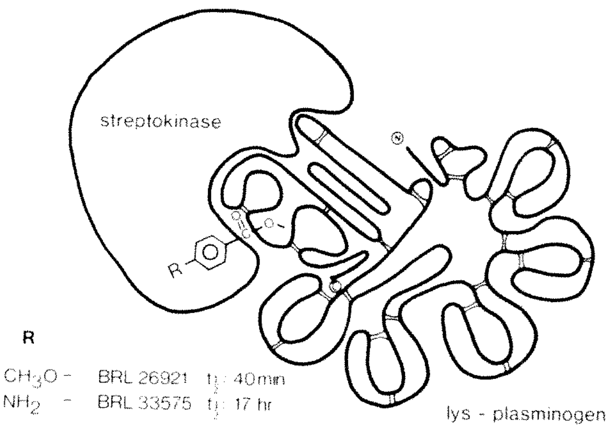

Fig 5. General structure of acylated streptokinase-plasminogen complexes as BRL 26921 and BRL 33575.

which assesses both the fibrinogen and the fragments X and Y, rather than with a coagulation rate assay.[148]

Acylated streptokinase–plasminogen complex. Stable acyl-derivatives of an equimolar streptokinase-plasminogen complex have been prepared by acylation of the Ser 740 residue located in the catalytic center of the light B-chain of plasminogen (BRL 26921 and BRL 33575, Beecham Pharmaceuticals, Surrey, UK)[149,150] (Fig 5). Because the catalytic center is functionally separate from its fibrin-binding site located in the heavy A-chain of plasminogen, acylated streptokinase-human plasminogen is catalytically inert so that it can circulate in the vascular system without reaction with either plasma inhibitors or plasminogen, but still bind to fibrin through the unmodified kringle domains of the plasminogen moiety. These compounds deacylate under physiological conditions following first-order kinetics, with half-lives of 40 minutes (*p*-anisoyl derivative BRL-26921) and 17 hours (*p*-aminobenzoyl derivative BRL-33575). The principle of a thrombolytic agent having the properties to bind to fibrin and a prolonged fibrinolytic effect has attracted considerable interest.

In a thrombotic model in the rabbit, acylated SK-plasminogen was significantly more thrombolytic than the unmodified SK-plasminogen complex, both given as bolus doses.[150] In a femoral vein thrombosis model in dogs, SK-plasminogen resulted in a deep fall to 20% of the original fibrinogen level at a dose that was not yet thrombolytic, whereas the two acylated compounds used at equivalent SK doses were thrombolytic but also markedly reduced the fibrinogen level.[151,152] In human volunteers, 5 mg of the BRL 26921 compound had little effect on the systemic fibrinolytic system; on a molar basis, this dose is equivalent to 178,000 IU streptokinase, which produces a significant decrease of fibrinogen and plasminogen.[153] At doses >5 mg, progressive reductions were observed in plasma levels of fibrinogen, plasminogen, and α_2-antiplasmin.

The clinical indication for which acylated SK-plasminogen complexes have been best studied is acute myocardial infarction. With an intracoronary bolus of 10 mg of BRL 26921 or above, angiographically determined reperfusion rates of 75% were obtained in 74 patients.[154] After IV bolus injection of 30 mg of BRL 26291, coronary reperfusion was achieved in 86% of 57 patients (Fig 6). Several minor bleeding episodes were reported in most cases and, with the latter dose, fibrinogen levels were reduced by as much as 80% of the preinfusion value.[155] Other side effects were those usually observed after streptokinase.[156] The main advantage of acylated SK-plasminogen complexes seems to be that they can be given as a single IV injection.

Single-chain urokinase-type plasminogen activator or pro-urokinase. By IV administration of only 3,000 IU of scu-PA or of urokinase per kilogram of body weight in dogs with an experimental thrombosis, Sumi and Colleagues[54] obtained complete thrombolysis within 90 minutes with scu-PA, whereas the lysis time was greater than three hours in the group treated with urokinase. Gurewich and coworkers[58] studied the thrombolytic effect of scu-PA of human kidney cell origin in rabbits and dogs with pulmonary embolus. In rabbits, the mean extent of thrombolysis after five hours was 6%, 17%, and 53% following infusion of saline, urokinase, and scu-PA, respectively. Infusion of urokinase was accompanied by systemic fibrinogenolysis, whereas scu-PA did not cause significant fibrinogen degradation. Dogs were found to be about ten times more sensitive to human urokinase than rabbits, but otherwise similar results were obtained as in rabbits.

The thrombolytic properties of recombinant scu-PA (rec-scu-PA), recombinant urokinase (rec-UK), and natural urinary urokinase (nat-UK) were compared[65] in rabbits with a radiolabeled thrombus in the jugular vein. The thrombolytic agents were infused IV over a period of four hours, and two hours later the extent of thrombolysis was measured as the difference between the radioactivity introduced in the clot and that recovered in the vein segment at the end of the experiment. Significant thrombolysis with nat-UK and rec-UK was only obtained with ≥240,000 IU/kg; this was associated with a marked systemic activation of the fibrinolytic system, as evidenced by consumption of plasminogen and α_2-antiplasmin and fibrinogen breakdown. Infusion of rec-scu-PA induced thrombolysis at a dose of ≥ 60,000 IU/kg without associated activation of the fibrinolytic system. However, t-PA induced a comparable degree of throm-

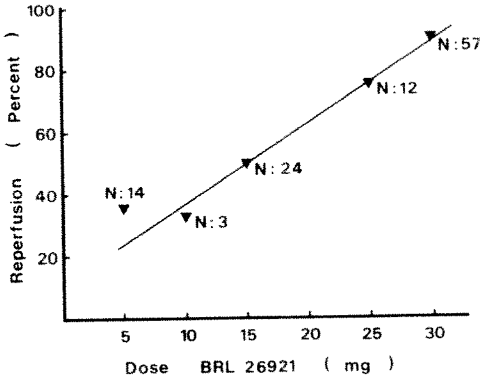

Fig 6. Reperfusion rates in patients with acute myocardial infarction given a single, intravenous injection of the acylated derivative of streptokinase-plasminogen, BRL 26921.[154]

bolysis without systemic activation of the fibrinolytic system at two- to fourfold lower concentrations. Although this difference could result from different reactivity of the rabbit toward both types of fibrinolytic agents, our findings obtained in a human plasma system in vitro[63] seem to support the conclusion that t-PA probably also has a higher specific thrombolytic activity in humans.

Intravenous infusion of scu-PA, isolated from a transformed kidney cell line (ACHN), at a rate of 20 μg per kilogram per minute for 30 minutes, induced within 23 minutes coronary thrombolysis in four dogs, without associated systemic fibrinolysis. Infusion of 10 μg per kilogram per minute of urokinase elicited coronary thrombolysis in four of seven dogs within 19 minutes but was associated with a decrease of fibrinogen to 43% of the preinfusion value.[66]

Scu-PA has been given IV in a 40 mg/kg dose over 60 minutes to six patients with acute myocardial infarction and occluded infarct-related coronary artery. Recanalization was obtained in four patients, as well as in one of the remaining two during subsequent administration of scu-PA directly into the coronary artery.[156] There was no systemic activation of the fibrinolytic system in five patients, but in one patient the fibrinogen level fell to 25% of the pretreatment level.

CONCLUSION

Despite very significant progress in the understanding of the biochemistry and the biological properties of thrombolytic agents over the last decade, thrombolytic treatment is not ingrained in medical practice. Most clinical experience is with the less fibrin-specific thrombolytic agents streptokinase and urokinase, administered IV or in the coronary circulation. The reluctance to use these first-generation thrombolytic agents in routine clinical practice is due to the risk and unpredictibility of bleeding and other, though minor, side effects, hesitation on the optimal dose regimen, uncertainty of the risk-benefit ratio and, in addition for urokinase, its price, which is several times higher than that of streptokinase. The first clinical trials indicate that an effective thrombolysis with t-PA can be obtained at a dose which does not induce significant systemic fibrinogenolysis in most patients. Clot-specificity of t-PA is relative, however, and depends on the dose regimen used. The efficacy and safety of t-PA and scu-PA, as compared with the thrombolytic agents of the first generation, are presently being studied in controlled clinical trials in the United States and in Europe.

REFERENCES

1. Mirsky IA, Perisutti G, Davis NC: The destruction of glucagon, adrenocorticotropin and somatotropin by human blood plasma. J Clin Invest 38:14, 1959

2. Kwaan HC, Astrup T: Fibrinolytic activity of reparative connective tissue. J Pathol Bacteriol 87:409, 1964

3. Janeway CA, Merler E, Rosen FS, Salmon S, Crain JO: Intravenous gamma-globulin. Metabolism of gamma-globulin fragments in normal and agamma-globulinemic persons. N Engl J Med 278:919, 1968

4. Peterson HI: Fibrinolysis and antifibrinolytic drugs in the growth and spread of tumours. Cancer Treat Rev 4:213, 1977

5. Cochrane CG, Revak SD, Wuepper WG: Activation of Hageman factor in solid and fluid phases. J Exp Med 138:1564, 1973

6. Unkeless JC, Tobia A, Ossowski L, Quigley JP, Rifkin DB, Reich E: An enzyme function associated with transformation of fibroblast by oncogenic viruses. J Exp Med 137:85, 1973

7. Unkeless JC, Gordon S, Reich E: Secretion of plasminogen activator by stimulated macrophages. J Exp Med 139:834, 1974

8. Brade V, Nicholson A, Bitter-Suermann D, Hadding V: Formation of the C-3 cleaving properdin enzyme on zymosan. J Immunol 113:1735, 1974

9. Astrup T: Cell-induced fibrinolysis: a fundamental process, in Reich E, Rifkin DB, Shaw E (eds): Proteases and Biological Control. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1975, p 343

10. Strickland S: Studies on the role of plasminogen activator in ovulation and early embryogenesis, in Magnusson S, Ottesen M, Foltman B, Danø K, Neurath H (eds): Regulatory Proteolytic Enzymes and Their Inhibitors. Oxford, Pergamon Press, 1978, pp 181–185

11. Virgi MAG, Vassali JD, Estensen RD, Reich E: Plasminogen activator of islets of Langerhans: Modulation by glucose and correlation with insulin production. Proc Natl Acad Sci USA 77:875, 1980

12. Heimark RL, Kurachi K, Fujikawa K, Davie EW: Surface activation of blood coagulation, fibrinolysis and kinin formation. Nature 286:456, 1980

13. Edgington TS: Activation of the coagulation system in association with neoplasia. J Lab Clin Med 96:1, 1980

14. Sloane BF, Dunn JR, Honn KV: Lysosomal cathepsin B: correlation with metastatic potential. Science 212:1151, 1981

15. Sottrup-Jensen L, Petersen TE, Magnusson S: Atlas of Protein Sequence and Structure, vol 5. 1978, p 91 (suppl 3)

16. Brockway WJ, Castellino FJ: Measurement of the binding of antifibrinolytic amino acids to various plasminogens. Arch Biochem Biophys 151:194, 1972

17. Wiman B, Collen D: Molecular mechanism of physiological fibrinolysis. Nature 272:549, 1978

18. Lijnen HR, Hoylaerts M, Collen D: Isolation and characterization of a human plasma protein with affinity for the lysine-binding sites in plasminogen. Role in the regulation of fibrinolysis and identification as histidine-rich glycoprotein. J Biol Chem 255:10214, 1980

19. Markus G, de Pasquale JL, Wissler FC: Quantitative determination of the binding of epsilon-aminocaproic acid to native plasminogen. J Biol Chem 253:727, 1978

20. Violand BN, Byrne R, Castellino FJ: The effect of alpha, omega-amino acids on human plasminogen structure and activation. J Biol Chem 253:5395, 1978

21. Hayes ML, Castellino FJ: Carbohydrate of the human plasminogen variants. I. Carbohydrate composition and glycopeptide isolation and characterization. J Biol Chem 254:8768, 1979

22. Bohmfalk JR, Fuller GM: Plasminogen is synthesized by primary cultures of rat hepatocytes. Science 209:408, 1980

23. Wallén P, Wiman B: Characterization of human plasminogen. II. Separation and partial characterization of different forms of human plasminogen. Biochim Biophys Acta 257:122, 1972

24. Robbins KC, Summaria L, Hsieh B, Shah RJ: The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem 242:2333, 1967

25. Groskopf WR, Summaria L, Robbins KC: Studies on the active center of human plasmin. Partial amino acid sequence of a peptide containing the active center serine residue. J Biol Chem 244:3590, 1969

26. Moroi M, Aoki N: Isolation and characterization of α_2-plasmin inhibitor from human plasma. A novel proteinase inhibitor which inhibits activator-induced clot lysis. J Biol Chem 251:5956, 1976

27. Wiman B, Collen D: Purification and characterization of

human antiplasmin, the fast-acting plasmin inhibitor in plasma. Eur J Biochem 78:19, 1977

28. Lijnen HR, Wiman B, Collen D: Partial primary structure of human α_2-antiplasmin. Homology with other plasma inhibitors. Thromb Haemost 48:311, 1982

29. Collen D: Identification and some properties of a new fast reacting plasmin inhibitor in human plasma. Eur J Biochem 69:209, 1976

30. Müllertz S, Clemmensen I: The primary inhibitor of plasmin in human plasma. Biochem J 159:545, 1976

31. Christensen U, Clemmensen I: Kinetic properties of the primary inhibitor of plasmin from human plasma. Biochem J 163:389, 1977

32. Wiman B, Collen D: On the kinetics of the reaction between human antiplasmin and plasmin. Eur J Biochem 84:573, 1978

33. Bennett WF: Two forms of tissue-type plasminogen activator (t-PA) differ at a single specific glycosylation site. Thromb Haemost 50:106, 1983

34. Rånby M: Tissue plasminogen activator. Isolation, enzymatic properties and assay procedure. Thesis Umeå University. Umeå, Medical Dissertations New Series, No 90, 1982

35. Pennica D, Holmes WE, Kohr WJ, Harkins RN, Vehar GA, Ward CA, Bennett WF, Yelverton E, Seeburg PH, Heyneker HL, Goeddel DV, Collen D: Cloning and expression of human tissue-type plasminogen activator cDNA in *E. coli.* Nature 301:214, 1983

36. Rijken DC, Collen D: Purification and characterization of the plasminogen activator secreted by human melanoma cells in culture. J Biol Chem 256:7035, 1981

37. Wallén P, Bergsdorf N, Rånby M: Purification and identification of two structural variants of porcine tissue plasminogen activator by affinity adsorption on fibrin. Biochim Biophys Acta 719:318, 1982

38. Rånby M, Bergsdorf N, Nilsson T: Enzymatic properties of the one- and two-chain form of tissue plasminogen activator. Thromb Res 27:175, 1982

39. Banyai L, Varadi A, Patthy L: Common evolutionary origin of the fibrin-binding structures of fibronectin and tissue-type plasminogen activator. FEBS Lett 163:37, 1983

40. Strassburger W, Wollmer A, Pitts JE, Glover ID, Tickle IJ, Blundell TL, Steffens GJ, Gunzler WA, Otting F, Flohé L: Adaptation of plasminogen activator sequences to known protease structures. FEBS Lett 157:219, 1983

41. Rånby M, Bergsdorf N, Pohl G, Wallén P: Isolation of two variants of native one-chain tissue plasminogen activator. FEBS Lett 146:289, 1982

42. Rijken DC, Hoylaerts M, Collen D: Fibrinolytic properties of one-chain and two-chain human extrinsic (tissue-type) plasminogen activator. J Biol Chem 257:2920, 1982

43. Chmielewska J, Rånby M, Wiman B: Evidence for a rapid inhibitor to tissue plasminogen activator in plasma. Thromb Res 31:427, 1983

44. Kruithof EKO, Tran-Thang C, Ransijn A, Bachmann F: Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. Blood 64:907, 1984

45. Verheijen JH, Mullaart E, Chang GTG, Kluft C, Wijngaards G: A simple, sensitive spectrophotometric assay for extrinsic (tissue-type) plasminogen activator applicable to measurements in plasma. Thromb Haemost 48:266, 1982

46. Verheijen JH, Chang GTG, Kluft C: Evidence for the occurrence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. Thromb Haemost 51:392, 1984

47. Wijngaards G, Groeneveld E: Temporarily increased inhibition by plasma of plasminogen activator activity in severely ill patients. Haemostasis 12:571, 1982

48. Juhan-Vague I, Moerman B, De Cock F, Aillaud MF, Collen D: Plasma levels of a specific inhibitor of tissue-type plasminogen activator (and urokinase) in normal and pathological conditions. Thromb Res 33:523, 1984

49. Paramo JA, Colucci M, Collen D, Van de Werf F: Plasminogen activator inhibitor in the blood of patients with coronary artery disease. Br Med J 291:573, 1985

50. Kruithof EKO, Ransijn A, Bachmann F: Inhibition of tissue plasminogen activator by human plasma, in Davidson JF, Bachmann F, Bouvier CA, Kruithof EKO (eds): Progress in Fibrinolysis, vol 6. Edinburgh, Churchill Livingstone, 1983, p 362

51. White WF, Barlow GH, Mozen MM: The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry 5:2160, 1966

52. Nielsen LS, Hansen JG, Skriver L, Wilson EL, Kaltoft K, Zeuthen J, Danø K: Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. Biochemistry 21:6410, 1982

53. Sumi H, Maruyama M, Matsuo O, Mihara H, Toki N: Higher fibrin-binding and thrombolytic properties of single polypeptide chain-high molecular weight urokinase. Thromb Haemost 47:297, 1982

54. Sumi H, Toki N, Sasaki K, Mihara H: Comparative properties of single and double polypeptide chains of high molecular weight urokinase, in Davidson JF, Bachmann F, Bouvier CA, Kruithof EKO (eds): Progress in Fibrinolysis, vol 6. Edinburgh, Churchill Livingstone, 1983, p 165

55. Wun TC, Ossowski L, Reich E: A proenzyme form of human urokinase. J Biol Chem 257:7262, 1982

56. Wun TC, Schleuning WD, Reich E: Isolation and characterisation of urokinase from human plasma. J Biol Chem 257:3276, 1982

57. Husain S, Gurewich V, Lipinski B: Purification and partial characterization of a single-chain high-molecular-weight form of urokinase from human urine. Arch Biochem Biophys 220:31, 1983

58. Gurewich V, Pannell R, Kelley P: Single-chain urokinase, a 55,000 MW zymogen precursor of urokinase with fibrin-dependent activation under physiological conditions. Haemostasis 14:126, 1984 (abstr)

59. Collen D, Zamarron C, Lijnen HR, Hoylaerts M: Activation of plasminogen by pro-urokinase. II. Kinetics. J Biol Chem 261:1253, 1986

60. Zamarron C, Lijnen HR, Collen D: Competitive inhibition by human plasma of the activation of plasminogen by pro-urokinase. Thromb Haemost 54:102, 1985 (suppl 1) (abstr)

61. Lijnen HR, Zamarron C, Collen D: Pro-urokinase: kinetics and mechanism of action. Thromb Haemost 54:118, 1985 (suppl 1) (abstr)

62. Heyneker HL, Holmes WE, Vehar GA: Preparation of functional human urokinase proteins. European Patent Application 83103629.8, Publication No 0092182A2. European Patent Office, Münich, FRG

63. Zamarron C, Lijnen HR, Van Hoef B, Collen D: Biological and thrombolytic properties of proenzyme and active forms of human urokinase. I. Fibrinolytic and fibrinogenolytic properties in human plasma in vitro of urokinase obtained from human urine or by recombinant DNA technology. Thromb Haemost 52:19, 1984

64. Gurewich V, Pannell R, Louie S, Kelley P, Suddith AL, Greenlee R: Effective and fibrin-specific clot lysis by a zymogen precursor form of urokinase (pro-UK). A study in vitro and in two animal species. J Clin Invest 73:1731, 1984

65. Collen D, Stassen JM, Blaber M, Winkler M, Verstraete M: Biological and thrombolytic properties of proenzyme and active forms of human urokinase. III. Thrombolytic properties of natural

and recombinant urokinase in rabbits with experimental jugular vein thrombosis. Thromb Haemost 52:27, 1984

66. Collen D, Stump D, Van de Werf F, Jang IK, Nobuhara M, Lijnen HR: Coronary thrombolysis in dogs with intravenously administered human pro-urokinase. Circulation 72:384, 1985

67. Podor TJ, Schleef RR, Loskutoff DJ: A competitive radioimmunoassay (RIA) for a fast acting inhibitor to plasminogen activator. Thromb Haemost 54:218, 1985 (suppl 1) (abstr)

68. Kosow DP: Kinetic mechanism of the activation of human plasminogen by streptokinase. Biochemistry 14:4459, 1975

69. Johnson AJ, McCarty WR: The lysis of artificially induced intravascular clots in man by infusions of streptokinase. J Clin Invest 38:1627, 1959

70. Alkjaersig N, Fletcher AP, Sherry S: The assay of urokinase preparation for contamination with thromboplastic moieties. J Lab Clin Med 65:732, 1965

71. Thorsen S: Differences in the binding to fibrin of native plasminogen and plasminogen modified by proteolytic degradation. Influence of ω-aminocarboxylic acid. Biochim Biophys Acta 393:55, 1975

72. Rákóczi I, Wiman B, Collen D: On the biological significance of the specific interaction between fibrin, plasminogen and antiplasmin. Biochim Biophys Acta 540:295, 1978

73. Aoki N, Sakata Y: Influence of α_2-plasmin inhibitor on adsorption of plasminogen to fibrin. Thromb Res 19:149, 1980

74. Hedner V, Nilson IM, Robertson B: Determination of plasminogen in clots and thrombi. Thromb Diath Haemorrh 16:38, 1966

75. Jacobson CD, Chandler AB: Thrombolysis in vitro. Scand J Clin Lab Invest 17:209, 1965, (suppl 84)

76. Ogston D, Ogston CM, Fullerton HW: The plasminogen content of thrombi. Thromb Diath Haemorrh 15:220, 1966

77. Chesterman CN, Allington MJ, Sharp AA: Relationship of plasminogen activator to fibrin. Nature 238:15, 1972

78. Aoki N, von Kaulla KN: Dissimilarity of human vascular plasminogen activator and human urokinase. J Lab Clin Med 78:354, 1971

79. Gurewich V, Hyde E, Lipinski B: The resistance of fibrinogen and soluble fibrin monomer in blood to degradation by a potent plasminogen activator derived from cadaver limbs. Blood 46:555, 1975

80. Ogston D, Bennett B, Mackie M: Properties of a partially purified preparation of a circulating plasminogen activator. Thromb Res 8:275, 1976

81. Binder BR, Spragg J, Austen KF: Purification and characterization of human vascular plasminogen activator derived from blood vessel perfusates. J Biol Chem 254:1998, 1979

82. Camiolo SM, Thorsen S, Astrup T: Fibrinogenolysis and fibrinolysis with tissue plasminogen activator, urokinase, streptokinase-activated human globulin and plasmin. Proc Soc Exp Biol Med 138:277, 1971

83. Thorsen S, Glas-Greenwalt P, Astrup T: Differences in the binding to fibrin of urokinase and tissue plasminogen activator. Thromb Diath Haemorrh 28:65, 1972

84. Wallén P: Activation of plasminogen with urokinase and tissue activator, in Paoletti R, Sherry S (eds): Thrombosis and Urokinase. London, Academic Press, 1977, p 91

85. Rånby M, Wallén P: A sensitive parabolic rate assay for the tissue plasminogen activator, in Davidson JF, Nilson IM, Åstedt B (eds): Progress in Fibrinolysis, vol 5. Edinburgh, Churchill Livingstone, 1981, p 233

86. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem 257:2912, 1982

87. Zamarron C, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by natural and recombinant tissue-type plasminogen activator. J Biol Chem 259:2080, 1984

88. Nieuwenhuizen W, Verheijen JH, Vermond A, Chang GTG: Plasminogen activation by tissue activator is accelerated in the presence of fibrin (ogen) cyanogen bromide fragment FCB-2. Biochim Biophys Acta 755:531, 1983

89. Kagitani H, Tagawa M, Hatanaka K, Ikari T, Saito A, Bando H, Okada K, Matsuo O: Expression in *E. coli* of finger-domain lacking tissue-type plasminogen activator with high fibrin affinity. FEBS Lett 189:145, 1985

90. Collen D: On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost 43:77, 1980

91. National Institutes of Health Consensus Development Conference: Thrombolytic therapy in thrombosis. Ann Intern Med 93:141, 1980

92. Marder VJ: The use of thrombolytic agents: Choice of patient, drug administration, laboratory monitoring. Ann Intern Med 90:802, 1979

93. Urokinase Pulmonary Embolism Trial Study Group: Urokinase pulmonary embolism trial. Phase 1 Results. A cooperative study. JAMA 214:2163, 1970

94. Urokinase Pulmonary Embolism Study Group: The Urokinase Pulmonary Embolism Trial: A national cooperative study. Circulation 47:1, 1973 (suppl II)

95. Urokinase-Streptokinase Pulmonary Embolism Trial Study Group: The Urokinase-Streptokinase Pulmonary Embolism Trial. Phase II. Results. JAMA 229:1606, 1974

96. Sharma GVRK, Burleson VA, Sasahara AA: Effect of thrombolytic therapy on pulmonary capillary blood volume in patients with pulmonary embolism. N Engl J Med 303:842, 1980

97. Groupe de Recherche Urokinase-Embolie Pulmonaire. Rapport préparé par B. Charbonnier de Tours: Etude multicentrique sur deux protocoles d'urokinase dans l'embolie pulmonaire grave. Arch Mal Coeur 7:773, 1984

98. Duckert F: Thrombolytic therapy. Semin Thromb Hemost 10:87, 1984

99. Trübestein G, Trübestein R, Ludwig M, Brecht Th, Harder Th: Fibrinolytic treatment with streptokinase and urokinase in deep vein thrombosis. Vasc Med 3:72, 1985

100. Goldhaber SZ, Buring JE, Lipnick RJ, Hennekens CH: Pooled analyses of randomized trials of streptokinase and heparin in phlebographically documented acute deep venous thrombosis. Am J Med 76:393, 1984

101. Widmer LK, Brandenberg E, Widmer MT, Schmitt HE, Duckert F, Marbet G, Ritz R: Late sequelae of deep venous thrombosis. Follow-up of 278 patients after anticoagulant and thrombolytic treatment. International Angiology 1:31, 1982

102. Rentrop KP: Thrombolytic therapy in patients with acute myocardial infarction. Circulation 71:627, 1985

103. Spann JF, Sherry S: Coronary thrombolysis for evolving myocardial infarction. Drugs 28:465, 1984

104. Yusuf S, Collins R, Peto R, Furberg C, Stampfer MJ, Goldhaber SZ, Hennekens CH: Intravenous and intracoronary fibrinolytic therapy in acute myocardial infarction: Overview of results on mortality, reinfarction and side-effects from 33 randomized controlled trials. Eur Heart J 6:556, 1985

105. Verstraete M: Intravenous administration of a thrombolytic agent is the only realistic therapeutic approach in evolving myocardial infarction. Eur Heart J 6:586, 1985

106. Bell WR: Streptokinase and urokinase in the treatment of pulmonary thromboemboli. Thromb Haemost 35:57, 1976

107. Bell WR, Meek AG: Guidelines for the use of thrombolytic agents. N Engl J Med 301:1266, 1979

108. Marder VJ: Guidelines for thrombolytic therapy of deep vein thrombosis. Prog Cardiovasc Dis 21:327, 1979

109. Aldrich MS, Sherman SA, Greenberg HS: Cerebrovascular complications of streptokinase infusion. JAMA 253:1777, 1985

110. Thayer CF: Results of postmarketing surveillance program on streptokinase. Curr Ther Res 30:129, 1981

111. Marbet GA, Eichlisberger R, Duckert F, Ritz R, da Silva MA, Biland L, Widmer LK, Schmitt E: Side effects of thrombolytic treatment with procine plasmin and low dose streptokinase. Thromb Haemost 48:196, 1982

112. Amery A, Deloof W, Vermylen J, Verstraete M: Outcome of recent thromboembolic occlusions of limb arteries treated with streptokinase. Br Med J 4:639, 1970

113. Verstraete M, Vermylen J, Schetz J: Biochemical changes noted during intermittent administration of streptokinase. Thromb Haemost 39:61, 1978

114. Rentrop KP, Blanke H, Karsch KR, Kreuzer M: Initial experience with transluminal recanalization of the recently occluded infarct-related coronary artery in acute myocardial infarction: comparison with conventionally treated patients. Clin Cardiol 2:92, 1979

115. Rentrop P, Blanke H, Karsch KR, Kaiser H, Köstering H, Leitz K: Selective intracoronary thrombolysis in acute myocardial infarction and unstable angina pectoris. Circulation 63:307, 1981

116. Cowley MJ, Hastillo A, Vetrovec GW, Fisher LM, Garrett R, Hess ML: Fibrinolytic effects of intracoronary streptokinase administration in patients with acute myocardial infarction and coronary insufficiency. Circulation 67:1031, 1983

117. Merx W, Dörr R, Rentrop P, Blanke H, Karsch KR, Mathey DG, Kremer P, Rutsch W, Schmutzler H: Evaluation of the effectiveness of intracoronary streptokinase infusion in acute myocardial infarction: Post-procedure management and hospital course in 204 patients. Am Heart J 102:1181, 1981

118. Timmis GC, Gangadharan V, Ramos RG, Hauser AM, Westveer DC, Stewart J, Goodfliesh R, Gordon S: Hemorrhage and the products of fibrinogen digestion after intracoronary administration of streptokinase. Circulation 69:1146, 1984

119. Hess H, Mietaschk A, Ingrisch H: Niedrig dosierte thrombolytische Therapie zur Wiederherstellung der Strombahne bei arterieller Verschlüssen. Dtsch Med Wochenschr 105:787, 1980

120. Hess H, Ingrisch H, Mietaschk A, Rath H: Local low-dose thrombolytic therapy of peripheral arterial occlusions. N Engl J Med 307:1627, 1982

121. Katzen BT, van Breda A: Low dose streptokinase in the treatment of arterial occlusion. Am J Roentgenol 136:1171, 1981

122. Miles CR, Blombery PA, Sacharias N, Stubbe JW, Bass CJ, Ferguson IA, Rosengarten DS, Stuckbery KE: The use of regional low dose streptokinase in recently thrombosed arterial graft. (submitted for publication)

123. Verstraete M, Vermylen J, Amery A, Vermylen C: Thrombolytic therapy with streptokinase using a standard dosage scheme. Br J Med 1:454, 1966

124. Sharma G, Cella G, Parisi A: Thrombolytic therapy. N Engl J Med 306:1268, 1982

125. Totty WG, Romano T, Benian GM, Gilula LA, Sherman LA: Serum sickness following streptokinase therapy. Am J Roentgenol 138:143, 1982

126. Weatherbee TC, Esterbrooks DJ, Katz DA, Aronow WS, Kenik JG, Muhiuddin SM: Serum sickness following selective intracoronary streptokinase. Curr Ther Res 35:433, 1984

127. Sheperd JT, Rao SS, Lawrence JR, Brodie MJ, Fulton WF: Streptokinase and serum enzymes. Br Med 1:562, 1976

128. Verstraete M, Vermylen J, Holleman W, Barlow GH: Biological effects of the administration of an equimolar streptokinase-plasminogen complex in man. Thromb Res 11:227, 1977

129. Matsuo O, Rijken DC, Collen D: Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature 291:590, 1981

130. Fletcher AP, Alkjaersig N, Sherry S, Genton E, Hirsh J, Bachmann F: The development of urokinase as a thrombolytic agent. Maintenance of a sustained thrombolytic state in man by its intravenous infusion. J Lab Clin Med 65:713, 1965

131. Latallo ZS, Lopaciuk S: New approach to thrombolytic therapy: the use of defibrase in connection with streptokinase. Thromb Diath Haemorrh 56:253, 1973 (suppl)

132. Duckert F, Muller G, Nyman D, Benz A: Treatment of deep vein thrombosis with streptokinase. Br Med J 1:479, 1975

133. Totty WG, Gilula LA, McClennan M, Ahmed P, Sherman L: Low-dose intravascular fibrinolytic therapy. Radiology 143:59, 1982

134. Korninger C, Matsuo O, Suy R, Stassen JM, Collen D: Thrombolysis with human extrinsic (tissue-type) plasminogen activator in dogs with femoral vein thrombosis. J Clin Invest 69:573, 1982

135. Collen D, Stassen JM, Verstraete M: Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. Effect of molecular form and dose of activator, age of thrombus and route of administration. J Clin Invest 71:368, 1983

136. Bergmann SR, Fox KAA, Ter-Pogossian MM, Sobel BE, Collen D: Clot selective coronary thrombolysis with tissue-type plasminogen activator. Science 220:1181, 1983

137. Van de Werf F, Bergmann SR, Fox KAA, De Geest H, Hoyng CF, Sobel BE, Collen D: Coronary thrombolysis with intravenously administered human tissue-type plasminogen activator produced by recombinant DNA technology. Circulation 69:605, 1984

138. Flameng W, Van de Werf F, Vanhaecke J, Verstraete M, Collen D: Coronary thrombolysis and infarct size reduction following intravenous infusion of recombinant tissue-type plasminogen activator in non-human primates. J Clin Invest 75:84, 1985

139. Van de Werf F, Ludbrook PA, Bergmann SR, Tiefenbrunn AJ, Fox KAA, De Geest H, Verstraete M, Collen D, Sobel BE: Coronary thrombolysis with tissue-type plasminogen activator in patients with evolving myocardial infarction. N Engl J Med 310:609, 1984

140. Sobel BE, Geltman EM, Tiefenbrunn AJ, Jaffe AS, Spadaro JJ, Ter-Pogossian MM, Collen D, Ludbrook PA: Improvement of regional myocardial metabolism after coronary thrombolysis with tissue-type plasminogen activator or streptokinase. Circulation 69:983, 1984

141. Collen D, Topol EJ, Tiefenbrunn AJ, Gold HK, Weisfeldt ML, Sobel BE, Leinbach RC, Brinker JA, Ludbrook PA, Yasuda I, Bulkley NH, Robison AK, Hutter AM Jr, Bell WR, Spadaro JJ Jr, Khaw BA, Grossbard EB: Coronary thrombolysis with recombinant human tissue-type plasminogen activator. A prospective, randomized placebo-controlled trial. Circulation 70:1012, 1984

142. TIMI-Study Group: The thrombolysis in myocardial infarction (TIMI) trial. Phase I findings. N Engl J Med 312:932, 1985

143. Verstraete M, Bernard R, Bory M, Brower RW, Collen D, de Bono DP, Erbel R, Huhmann W, Lennane RJ, Lubsen J, Mathey D, Meyer J, Michels HR, Rutsch W, Schartl M, Schmidt W, Uebis R, von Essen R: Randomised trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. Lancet 1:842, 1985

144. Verstraete M, Bleifeld W, Brower RW, Charbonnier B, Collen D, de Bono DP, Dunning AJ, Lennane RJ, Lubsen J, Mathey DG, Michel PL, Raynaud Ph, Schofer J, Vahanian A, Vanhaecke J,

van de Kley GA, Van de Werf F, von Essen R: Double-blind, randomised trial of intravenous tissue-type plasminogen activator versus placebo in acute myocardial infarction. Lancet 2:965, 1985

145. Verstraete M, Bounameaux H, De Cock F, Van de Werf F, Collen D: Pharmacokinetics and systemic fibrinogenolytic effects of recombinant human tissue-type plasminogen activator (rt-PA) in man. J Pharmacol Exp Ther 235:506, 1985

146. Collen D, Bounameaux H, De Cock F, Verstraete M: Analysis of coagulation and fibrinolysis during intravenous infusion of recombinant human tissue-type plasminogen activator (rt-PA) in patients with acute myocardial infarction. Circulation 73:511, 1986

147. Rampling MW, Gaffney PJ: The sulphite precipitation method for fibrinogen measurement: Its use on small samples in the presence of fibrinogen degradation products. Clin Chim Acta 67:43, 1976

148. Vermylen C, De Vreker R, Verstraete M: A rapid method for assay of fibrinogen fibrin polymerization time. Clin Chim Acta 8:418, 1963

149. Smith RAG, Dupe RJ, English PD, Green J: Fibrinolysis with acyl enzymes: A new approach to thrombolytic therapy. Nature 290:505, 1981

150. Smith RAG, Dupe RJ, English PD, Green J: Acyl-enzymes as thrombolytic agents in rabbit model of venous thrombosis. Thromb Haemost 47:269, 1982

151. Dupe RJ, English PD, Smith RAG, Green J: The activity of acylated strepokinase-plasminogen complex (BRL 26921) in dog models of thrombosis, in Davidson JF, Bachmann F, Bouvier CA, Kruithof EKO (eds): Progress in Fibrinolysis, vol VI. Edinburgh, Churchill Livingstone, 1983, p 240

152. Dupe RJ, English PD, Smith RAG, Green J: Acyl-enzymes and thrombolytic agents in dog models of venous thrombosis and pulmonary embolism. Thromb Haemost 51:248, 1984

153. Prowse CV, Hornsey V, Ruckley CV, Bolton FE: A comparison of acylated streptokinase-plasminogen complex and streptokinase in healthy volunteers. Thromb Haemost 47:132, 1982

154. Harris GS, Smith RAG, Dupe RJ: Acyl-enzymes: A novel class of thrombolytic agents, in Collen D, Lijnen HR, Verstraete M (eds): Thrombolysis: Biological and Therapeutic Properties of New Thrombolytic Agents. Edinburgh, Churchill Livingstone, 1985

155. Lämmle B, Duckert F, Noll G, Lohri A, Marbet GA, Huber P, Biland L, Schmitt HE: Thrombolyse mit acylierten Streptokinase-Plasminogen-Komplexen (BRL 26.921 und BRL 33.375). Bern, 3 Kongress für Thrombose und Blutstillung, February 1984 (in press)

156. Van de Werf F, Nobuhara M, Collen D: Coronary thrombolysis with human single chain urokinase-type plasminogen activator (scu-PA) in patients with acute myocardial infarction. Ann Intern Med (in press)

Establishment and Characterization of a Human Plasma Cell Myeloma Culture Having a Rearranged Cellular *myc* Proto-oncogene

By Adi F. Gazdar, Herbert K. Oie, Ilan R. Kirsch, and Gregory F. Hollis

Using a serum-free defined medium, we have established a human cell line, NCI-H929, from a malignant effusion occurring in a patient with IgAk myeloma. The cultured cells have the morphologic, ultrastructural, biochemical, immunologic, and cytochemical features of plasma cells. The cells have rearranged alpha and kappa genes and synthesize and secrete high amounts of IgAk ($>80\ \mu g/10^6$ cells per 24 hours). The cells express surface immunoglobulin (alpha and kappa), the plasma cell antigen PCA-1, the transferrin receptor (T9) and T10 but lack antigens associated with earlier stages of B cell development (HLA-DR, B1, B2, B4, CALLA), as well as other leukocyte-macrophage antigens and Epstein-Barr virus (EBV) nuclear antigen. Although molecular studies confirm that both the tumor and cultured cells are derived from the same clone of malignant B cells, the tumor cells were predominantly near-diploid, whereas the cultured cells are predominantly near-tetraploid with six copies of chromosome 8, four to six of which have an 8q+ abnormality. However, both the tumor and the cultured cells have a rearrangement of the cellular *c-myc* proto-oncogene (located at 8q24) and express *c-myc* RNA. Although a modest number of human "plasmacytoid" cell lines have been established, most are lymphoblastoid lines lacking plasma cell features, while others appear to be early secretory cells. In contrast, NCI-H929 is a differentiated, highly secretory human plasma cell line.

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DESPITE INTENSE EFFORTS in many laboratories during the past 20 years, plasma cell (PC) myelomas and leukemias have been one of the most difficult of human malignancies to establish in continuous culture.[1] Although a modest number of "plasmacytoid" cell lines have been reported, the vast majority of these are lymphoblastoid cell lines (LCLs), which result from in vitro transformation of nonmalignant B cells by Epstein-Barr virus.[2] In contrast, many mouse plasmacytoma and human Burkitt lymphoma (BL) lines exist.[3-7] In both of these otherwise very different B cell tumors, specific reciprocal translocations occur that bring the cellular *myc* proto-oncogene (*c-myc* gene) and an immunoglobulin (Ig) gene segment into the same chromosomal region.[3-6] These events result in the preferential transcription, and perhaps abnormal regulation, of the translocated *c-myc* gene. Although similar molecular events probably occur in rat PC tumors,[8] human myelomas have not been associated with rearrangement or inappropriate transcription of *c-myc,* nor with structural abnormalities of its chromosomal location (8q24).[9]

Normal and malignant PCs represent the final, terminally differentiated, highly secretory stage of B cell differentiation. They have several characteristic features, including typical morphology, ultrastructure, cytochemistry, the presence of functional rearranged heavy- and light-chain genes, secretion of monoclonal Ig, a low proliferative cell fraction, loss of most B cell antigens, and expression of the PC-associated antigen PCA-1.[10-18]

In this report we describe the establishment of a cell line, NCI-H929, from a malignant effusion occurring in a patient with IgAk myeloma.The patient's tumor and cultured cells have the morphological and antigenic properties of fully differentiated malignant PCs, lack EBV nuclear antigen (EBNA), and secrete high concentrations of IgAk. Of major interest, a rearrangement of the *c-myc* oncogene is present in both the tumor and the cultured cells, in association with an addition to the long arm of chromosome 8 (8q+).

MATERIALS AND METHODS

Cell culture and characterization. The NCI-H929 cell line was initiated from a malignant pleural effusion. The cells at the interface of a discontinuous Ficoll-Hypaque gradient were harvested, washed, resuspended in various media in 75-cm^2 flasks at a density of about 1×10^6/mL and incubated in a humidified 6% CO$_2$ atmosphere at 37 °C. Cytological examination of the interface cells indicated that more than 90% consisted of malignant PCs. The media used included RPMI 1640 supplemented with 10% fetal bovine serum or 10% to 20% patient's pleural fluid, and various serum-free media developed in our laboratory for the selective growth of lung cancer. The latter included ACL-3 formulated by Dr M. Brower for the growth of adenocarcinomas and modified by us.[19] ACL-3 consists of RPMI 1640 medium supplemented with insulin (20 μg/mL), transferrin (10 μg/mL), sodium selenite (2.5×10^{-8} mol/L), hydrocortisone (5×10^{-8} mol/L), epidermal growth factor (1 ng/mL), ethanolamine/phosphorylethanolamine (1×10^{-5} mol/L of each), triiodothyronine (1×10^{-10} mol/L), bovine albumin (0.2%), sodium pyruvate (0.5 mmol/L), HEPES buffer (10 mmol/L), and extra glutamine (2 mmol/L). Mycoplasma contamination was tested for by liquid and agar plate culture techniques and by the Hoechst stain method by Microbiological Associates, Bethesda, Md. Species identification was by isoenzyme analysis, using agarose electrophoresis (Authentikit apparatus and reagents, Corning Medical, Medfield, Mass).

Morphological studies. Air-dried cytospins of cultured cells were stained with Wright-Giemsa or various special stains. For transmission electron microscopy (performed by Dr B. Kramarsky, Electronucleonics, Silver Spring, Md), cell pellets were fixed in 2.5% glutaraldehyde, postfixed in chrome-osmium, and stained with uranyl acetate. Thin sections were examined in a Siemens Elmiskop 1A electron microscope.

DNA index and cytogenetic studies. The DNA index (DI) of tumor and cultured cells was determined by flow cytometry using an EPICS V fluorescence-activated cell sorter (Coulter Electronics, Hialeah, Fa) after propidium iodide staining.[20] For chromosome analysis (performed by P. Berry, Litton-Bionetics, Kensington, Md),

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cells were treated with colchicine, lysed with hypotonic solution, and stained with trypsin-Giemsa.

Immunoglobulin and antigen assays. Cell lysates and clarified culture supernatant fluids were tested for the presence of Ig by double immunodiffusion assays, using polyclonal antibodies to human heavy and light chains. Cytoplasmic Ig was tested for by an immunoperoxidase method. Ig concentrations in cell lysates and fluids were quantitated by rate nephelometry with a Beckman immunocytochemistry Analyzer II.[21] The presence of nuclear terminal deoxynucleotidyl transferase was determined by an immunoperoxidase method. EBNA was tested for as described previously.[7] Surface antigens were tested for by incubating cells with saturating concentrations of fluorescein-labeled or unlabeled monoclonal antibodies (10^6 cells in 100 μL at 4 °C for 30 minutes), washed twice, and, if the primary antibody was unlabeled, incubated with fluoresceinated goat antimouse Ig of the appropriate class, washed twice, and analyzed for log fluorescence intensity using an EPICS V cell sorter. The sources of the monoclonal antibodies were as follows: Tac from Dr T. Waldman, National Cancer Institute; kappa and lambda from Cappel Laboratories, Cochranville, Penn; T9 and T10 from Ortho Diagnostics, Raritan, NJ; B1, B2, B4, CALLA, and I2 from Coulter Immunology, Hialeah, Fla; and the others from Becton Dickinson Laboratory, Sunnyvale, Calif.

Other cell lines. The antigenic properties of NCI-H929 cells were compared with those of other B cell lineage cultures: Lymphoblastoid cell lines (LCLs) (BL-2, GM 1500, CB23, LAZ409, UC729-HF$_2$, and L1CR-LON-LMy2) and BL cell lines[7] (ST-486, KK124, Raji, Daudi, Ramos, and CW678) were obtained from Drs Ian Magrath (NCI, Bethesda, Md), Michael Kuehl (NCI), and Steven Giardino (PRI, Frederick, Md) or initiated by us. "Plasmacytoid" cell lines U266, RPMI 8226, HS Sultan, and ARH-77[22-25] were obtained from the American Type Culture Collection, Rockville, Md.

DNA and RNA extraction and analysis. DNA was prepared as previously described[26] from the NCI-H929 cell line, directly from the cells of the pleural effusion after harvesting them from a discontinuous Ficoll-Hypaque gradient, and from a control cell line of T cell origin. The genomic DNAs were digested with *Eco*RI, fractionated on a 0.8% agarose gel (10 μg per lane), and transferred to nitrocellulose paper, according to the method of Southern.[27] RNA extracts and blots were performed as previously described.[28] DNA and RNA blots were hybridized to ^{32}P-labeled human DNA probes corresponding to (a) Ig mu probe, a 1.3-kilobase (kb) genomic *Eco*RI–*Eco*RI fragment containing exons for the first two coding domains of the mu constant region[29]; (b) Ig alpha probe, a genomic 4.2-kb *Xho*I–*Eco*RI fragment containing the alpha constant region (the latter site being donated by the plasmid pBR322)[29]; (c) Ig kappa probe, a 2.5-kb genomic *Eco*RI–*Eco*RI fragment containing the constant region[30]; and (d) *c-myc* probe, a 1.7-kb *Cla*I–*Eco*RI fragment containing exon 3.[31] The transfers were washed at 55 °C in 15 mmol/L NaC1/1.5 mmol/L sodium citrate, pH 7.0, containing 0.1% $NaDodSO_4$ and visualized by autoradiography.

RESULTS

Case history. A 62-year-old white woman was diagnosed as having IgAk PC myeloma. Her bone marrow was virtually replaced with immature PCs (Fig 1A), and she had a serum IgA level of 9 g/dL. She was treated with vincristine, melphalan, cytoxan, and prednisone and achieved a brief clinical response. At relapse, nine months after diagnosis, a right pleural effusion was sent to our laboratory for diagnostic workup. The patient died a month later, and autopsy revealed widespread infiltration of the viscera, including lungs and pleura, with malignant PCs, many of which had bizarre or multinucleated forms (Fig 1B).

Establishment of cell culture. Gradient-concentrated cells from the malignant effusion were cultured in various media. No significant growth occurred in media supplemented with bovine serum or clarified effusion fluid. In contrast, immediate and continuous growth occurred in fully defined ACL-3 medium, with establishment of cell line NCI-H929. The cultured cells have a population doubling time of about 50 hours and grow as irregular floating cells, either singly or in small, loose clusters. Giant forms are frequent, some of which are multinucleated. Cell size varies from 20 to more than 50 μm. The cell line is free of *Mycoplasma* contamination.

Morphological studies. Wright-Giemsa–stained cultured cells (Fig 1C) resemble the abnormal plasma cells present at autopsy. Ultrastructural examination (Fig 2) confirmed that the cells are immature PCs, with abundant rough endoplasmic reticulum arranged in perinuclear concentric lamellae and a prominent golgi present as multiple paranuclear apparati. Many mitochondria are present, free ribosomes are sparse, nucleoli are prominent, and the chromatin clumped. Cytochemical stains indicated that the

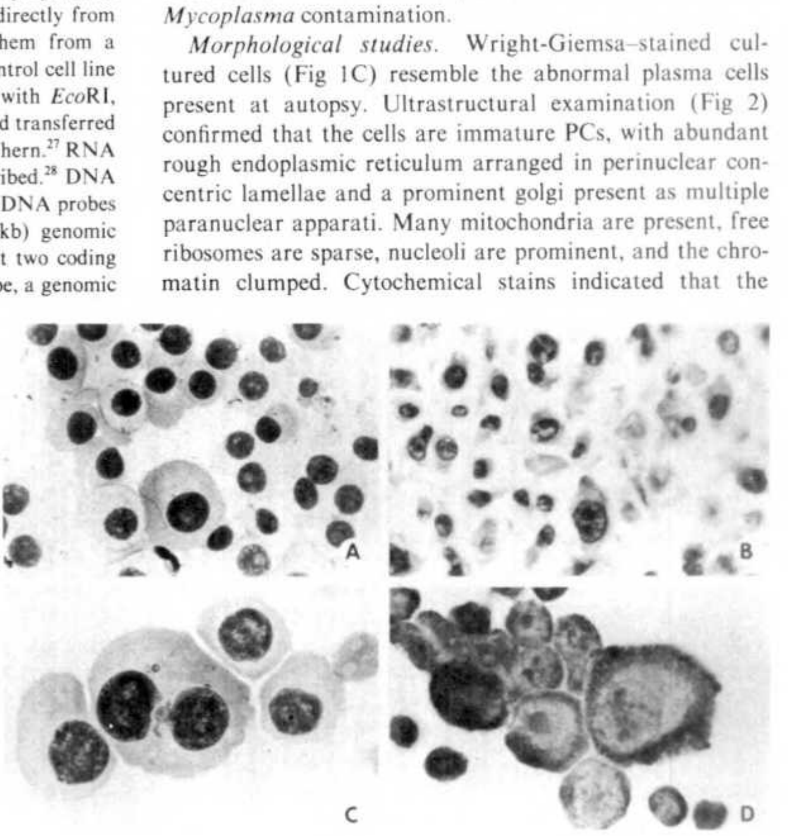

Fig 1. Morphological appearances of NCI-H929 tumor and cultured cells. (A) Bone marrow aspirate at diagnosis. Almost all the cells are nucleated PCs, with occasional giant forms. Wright-Giemsa. (B) Histological section of right pleura at autopsy. The cellular infiltrate consists predominantly of abnormal PCs with many hyperchromatic and bizarre forms. Hematoxylin and eosin. (C) Appearance of cultured cells. The large cells are plasmacytoid, with prominent nucleoli, occasional giant or multinucleated forms, and perinuclear clear areas (hof). Wright-Giemsa. (D) Immunocytochemical demonstration of kappa light chain. The staining is predominantly cytoplasmic with sparing of the hof areas. Immunoperoxidase technique. Original magnifications ×300; current magnifications ×192.

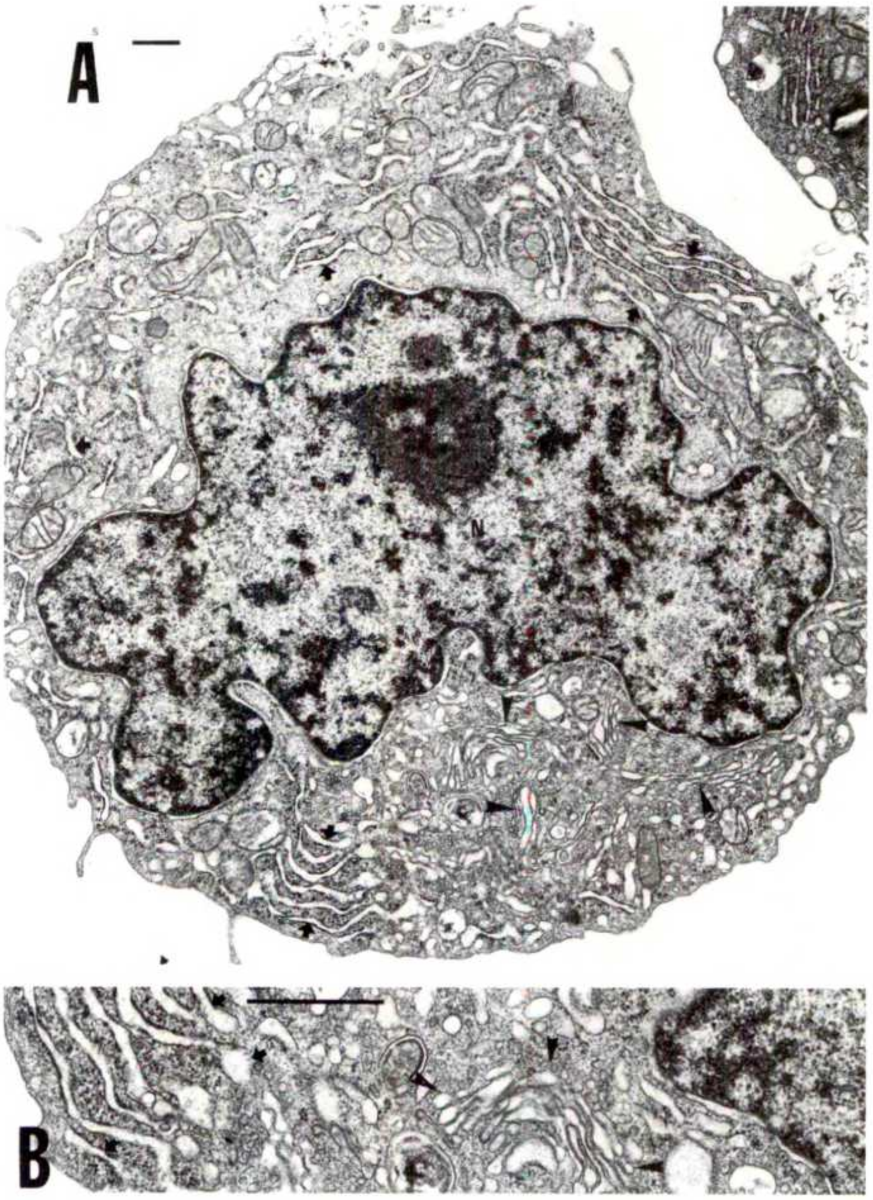

Fig 2. (A) Ultrastructure of NCI-H929 cell line. The appearance is that of an immature PC with large nucleus having a prominent nucleolus and clumped chromatin. The rough endoplasmic reticulum is extensive and arranged as perinuclear lamellae (arrows). The golgi is well developed (arrowheads). Insert (B) is a higher magnification of the cytoplasmic features. Bars represent 1 μmol/L.

tumor and cultured cells stain brightly with methyl green pyronin, acid phosphatase, alpha naphthyl acetate esterase, and beta glucuronidase, but not with periodic-acid Schiff.

DNA index and cytogenetic studies. DNA histograms (Fig 3) of the gradient-concentrated pleural fluid cells indicated that almost all the cells were aneuploid, with 90% having a DI of 1.15 (peak 1) and 10% a DI of 2.3 (peak 2). After culture, peak 2 became the dominant population (Fig 3). Cytogenetic studies of the cell line at day 87 of culture (Fig 4) indicated that approximately 80% of the metaphases consisted of near tetraploid cells (90 to 92 chromosomes) with a minor near-diploid subpopulation (44 to 48 chromosomes). Banding studies demonstrated a human karyotype with multiple structural abnormalities (Fig 4). Of particular interest, six copies of two chromosomes (numbers 1 and 8) were present in the near-tetraploid cells. Additional chromosomal material of unidentified origin was attached to the long arm of chromosome 8 (8q+) in at least four, and usually in all six, copies. All six copies of chromosome 1 were abnormal, one pair demonstrating an apparent duplication of the long arm, dup(1)(q11–25), and the other four having a deletion of the short arm of variable length.

Ig and antigen studies. Immunodiffusion studies demonstrated that the pleural fluid, culture medium, and extracts of cultured cells contained IgAk. The presence of intracytoplasmic Ig was confirmed by immunoperoxidase stain (Fig 1D). Quantitative studies demonstrated that the cultured cells were secreting 83 μg/10^6 cells per 24 hours and contained 7.1 μg/10^6 cells of intracytoplasmic IgA. These high concentrations were confirmed by an enzyme-linked immunosorbent assay. DNA and RNA from the tumor and cell lines were analyzed with ^{32}P-labeled probes derived from the Ig constant regions (Fig 5). Patterns from both samples were the same: one germline and one identically rearranged

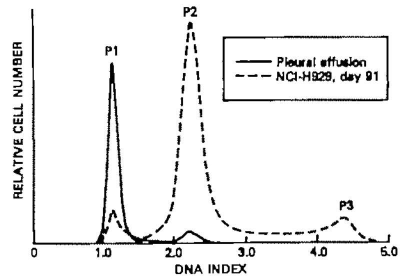

Fig 3. DNA histograms of NCI-H929 malignant effusion and 91-day-old culture. In both specimens, all, or nearly all, of the cells are aneuploid. Of the tumor cells, 90% had a DNA index (DI) of 1.15 (peak 1) and 10% a DI of 2.3 (peak 2). In the cell culture, peak 2 is the major component (77%) and peak 1 relatively small (8%). In addition, a third peak (peak 3) is present (15%), presumably representing the dividing (G_2 and M) cells of peak 2.

alpha and kappa genes were noted, while both mu genes were deleted. Abundant alpha and kappa mRNA were present, confirming the Ig production data (Fig 5).

Flow cytometric studies of both tumor and cultured cells demonstrated the presence of alpha and kappa surface immunoglobulin (2+), PcA-1 (3+) and T10 (2+) antigens, and absence of other B cell antigens (B1, B2, B4, CALLA, I2, and HLA-DR). Representative antigen expression patterns are illustrated in Fig 6. In addition, the following lymphocyte and macrophage associated antigens also were absent: L1, L2, L3, L4, L7, L8, L9, L11, L12, LeuM1, LeuM3, and TAC. The cells lacked expression of terminal deoxynucleotidyl transferase and EBNA. The transferrin receptor (T9 antigen) was not detected on the tumor cells, but was present on the cultured cells.

The antigen expression patterns of other B cell lines were compared to those of NCI-H929 (Table 1). The B lymphoblastoid and BL lines always expressed very high concentrations of HLA-DR, consistent expression of B1 and B4, and occasional expression of B2, CALLA, and T10. In addition, some lines expressed low levels of PCA-1. EBNA was present in all LCL and some BL lines. The 'plasmacytoid' lines demonstrated considerable heterogeneity. The HS Sultan and ARH 77 lines were EBNA positive and their surface antigen patterns indistinguishable from those of LCLs. U266 cells were EBNA negative and expressed PCA-1 and HLA-DR, but lacked other B antigens. RPMI 8226 lacked EBNA and HLA-DR, but expressed moderate amounts of T10 and PCA-1 and low amounts of B1 and B2.

Genomic blot analysis. We examined the state of the *c-myc* gene in the patient's tumor and cultured cells. This analysis (Fig 7) revealed germline and rearranged bands (12.0 and 14.5 kb, respectively) in both the tumor and the

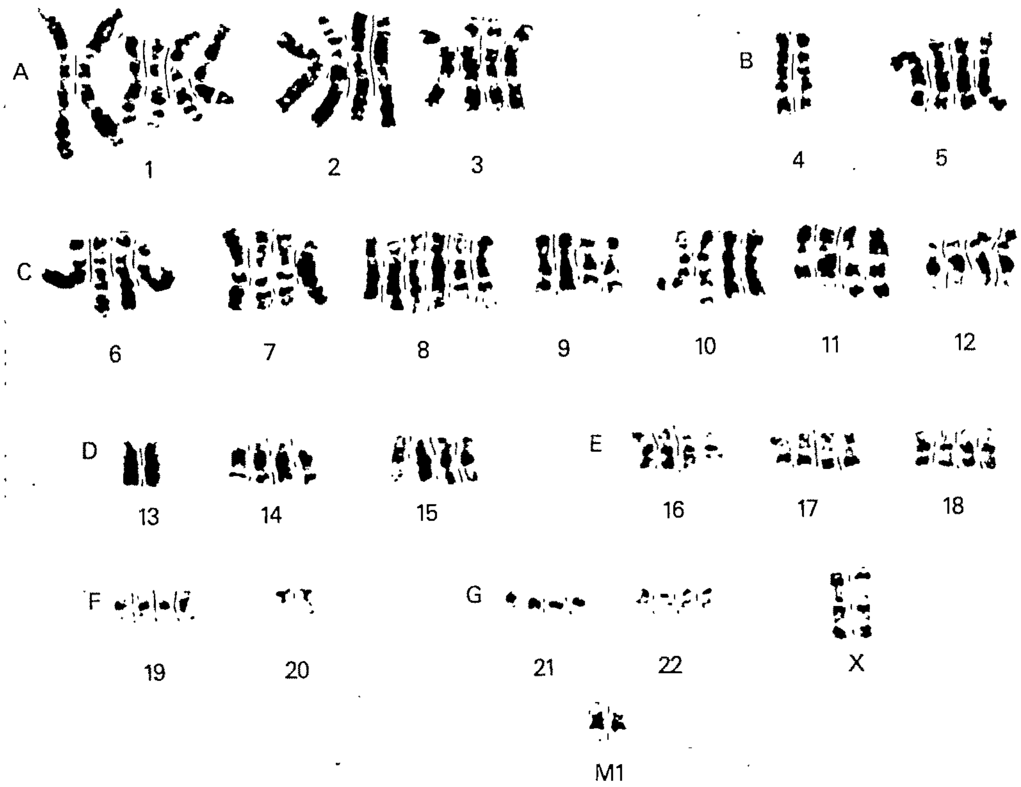

Fig 4. Representative G-banded karyotype of NCI-H929 cells, 87 days after initiating culture. All six copies of chromosome 1 are abnormal; the two illustrated on the left have an apparent duplication of the long arm, while the other four copies have deletions of the short arm of varying size. Of the six copies of chromosome 8, the one on the left is normal appearing; four and perhaps all five of the other copies have an 8q+ abnormality. Other consistent abnormalities included t(10q, 12p) in two of four copies, del (12p) in the other two copies of chromosome 12, and a marker chromosome (M1), possibly i(16q). Analysis: 90, XX.

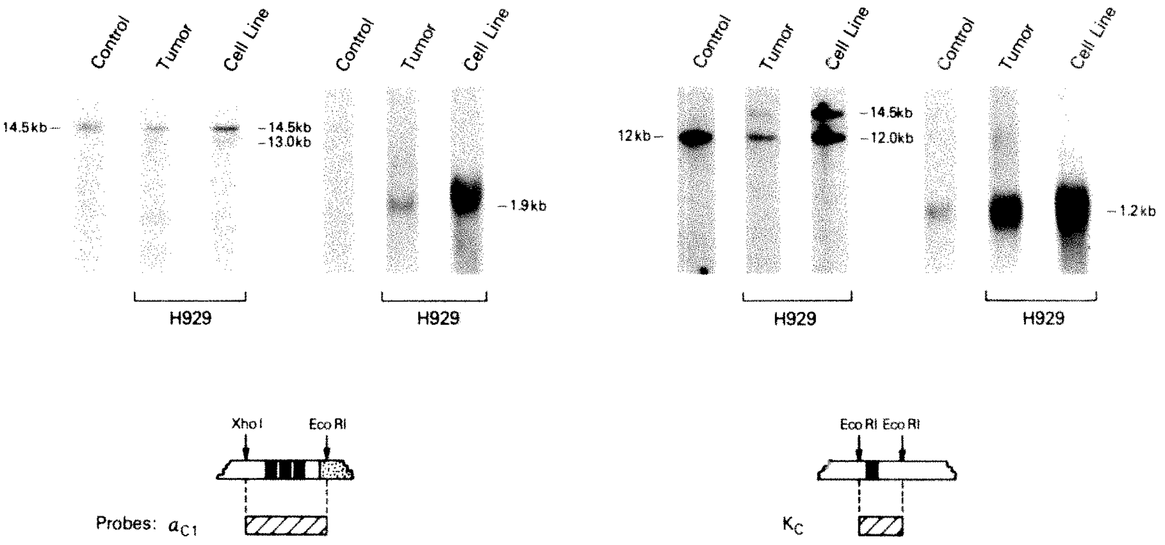

Fig 5. Ig gene expression and rearrangement of NCI-H929 tumor and cell line. The figure consists of 12 lanes, which are numerically referred to sequentially from left to right. Three DNA samples (lanes 1 through 3), a control T cell line, NCI-H929 tumor and cell line, are shown in a *BamHI* Southern genomic blot analysis probed with human alpha constant region-one. In all three DNAs, the germ line 14.5-kb band and in NCI-H929 tumor and cell line a rearranged 13.0-kb band is identified. (Note: the 13.0-kb band is faint in NCI-H929 tumor.) In lanes 4 through 6, analysis using the same probe is shown on three RNA samples, control (KK124, a mu and kappa producing Burkitt lymphoma cell line) and NCI-H929 tumor and cell line. Both NCI-H929 tumor and cell line synthesize a 1.9-kb Ig alpha mRNA. In lanes 7 through 9, three DNA samples, a control T cell line, and NCI-H929 tumor and cell line were digested with *BamHI* restriction endonuclease and probed with human kappa constant region. All three DNAs contain the germ line 12.0-kb band, and in NCI-H929 tumor and cell line, a 14.5-kb rearranged band is identified. Lanes 10 through 12 present a Northern blot analysis using a kappa constant probe that identifies a 1.2-kb mRNA in control (KK124) and NCI-H929 tumor and cell line RNA.

cell line, demonstrating that a DNA rearrangement had occurred near the *c-myc* gene. In the tumor cells, the germ line band was of greater intensity than the rearranged one, while in the cultured cells, both bands were of approximately the same intensity. To determine whether the rearranged band reflected an *Eco*RI restriction enzyme polymorphism, we digested the DNA samples with the restiction enzymes *Hind*III, *Sst*I, and *Xba*I. In all cases, a rearranged band was seen (data not shown), ruling out a simple restriction site polymorphism. *c-myc* RNA was present in both the tumor and the cultured cells as determined by Northern analysis (data not shown).

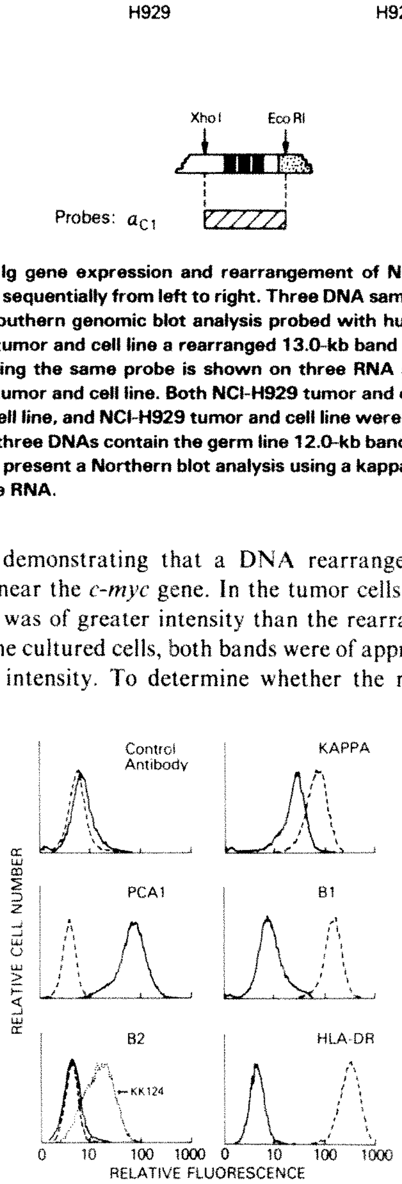

Fig 6. Expression of surface antigens on PC line NCI-H929 and BL line ST 486. NCI-H929 lacks expression of B1, B2, and HLA-DR and has weak expression of kappa light chain and strong expression of PCA-1. ST 486 lacks expression of B2 and PCA-1 and has moderate expression of kappa light chain and strong expression of HLA-DR. Because ST 486, an EBNA-negative BL line, lacks expression of B2, an epitope of the common CR2 and EBV receptor, B2 expression by KK124, an EBNA-positive BL line, is demonstrated. ——, NCI-H929; ---ST 486.

DISCUSSION

In this report, we describe the establishment, using serum-free defined medium, of a human PC culture. The cell line has the characteristic morphology, ultrastructure, and cytochemical features of PCs.[32] It secretes high concentrations of the same Ig class (IgAk) as did the patient's tumor cells, more than tenfold higher than those reported for other human lines. Identical Ig gene rearrangements were detected in the tumor and cultured cells, confirming that both were derived from the same clone of malignant B cells[33] and provide a molecular signature for the cell line.

To determine the relationship of NCI-H929 cells and other human "plasmacytoid" lines to LCL and BL lines, both of which may secrete Ig,[2,34] we compared their surface expression patterns of B cell specific and associated antigens. Most normal and malignant B cells, other than PCs, express B1, B4, and Ia-like antigens (HLA-DR and I2), while B2 and CALLA have more stage-restricted expression patterns.[11-16] PCs and myeloma cells lack expression of these antigens but acquire expression of a plasma cell-associated antigen (PCA-1) and T10, an antigen present on various activated lymphocytes.[14-16] PCs and myeloma cells lack

Table 1. Antigen Expression on B Cell Lines and NCI-H929 Tumor and Cultured Cells

	Antigen (No. Positive/No. Tested)								
Cell Line Type	B1	B2	B4	CALLA	HLA-DR	PCA-1	T9	T10	EBNA
B lymphoblastoid	3+	1+	1−2+	1−2+	3−4+	1+	NT	1+	Pos.
(n = 6)	(6/6)	(4/6)	(6/6)	(3/6)	(6/6)	(3/6)		(1/2)	(3/3)
Burkitt lymphoma	2−3+	1−2+	1−3+	−	3−4+	1+	NT	1−2+	Pos.
(n = 6)	(6/6)	(2/6)	(6/6)	(0/6)	(6/6)	(2/6)		(3/6)	(3/6)
"Plasmacytoid"									
HS Sultan	2+	1+	1+	1+	3+	1+	NT	1+	Pos.
ARH 77	3+	2+	2+	−	3+	1+	NT	−	Pos.
U266	−	−	−	−	3+	2+	NT	NT	−
RPMI 8226	1+	1+	−	−	−	2+	NT	2+	−
NCI-H929 tumor cells	−	−	−	−	−	3+	−	2+	NT
NCI-H929 cell line	−	−	−	−	−	3+	2+	2+	−

EBV nuclear antigen (EBNA) was determined as previously described[7] and scored as positive or negative. Surface antigen expression was determined by flow cytometry using monoclonal antibodies as described in the text. In all positive examples, >50% of the cells expressed the antigen. The degree of positive expression (1−4+) is reported as illustrated by Anderson et al.[15] All five "plasmacytoid" cell lines expressed human forms only of the enzymes purine nucleoside phosphorylase, peptidase B, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase (all lines homozygous for the slow moving B form). Pos., positive; −, no detectable expression; NT, not tested.

receptors for EBV (B2 antigen)[35] and are EBNA negative.[1,6] The antigen expression patterns of NCI-H929 tumor and cultured cells were identical, except for the absence of the transferrin receptor, T9,[36] on the former. Myeloma tumors have a low proliferative component.[18] Expression of T9 is associated with dividing cells, including mouse myeloma cells,[37] and it is present on the cultured cells that have a much higher proliferative fraction than the tumor cells. Both the tumor and the cultured cells had antigen patterns characteristic of fully mature PCs and could readily be distinguished from LCL and BL lines.

Although approximately 12 human "plasmacytoid" cell lines have been reported in the literature, most lack secretory and other PC features and many appear to be EBNA-positive LCLs.[38-42] Four of the LCLs we tested (ARH-77, HS Sultan, GM 1500, and L1CR-LON-HMy 2) were started from myeloma patients and were originally (or currently) described as PC lines. U266 cells expressed both HLA-DR and PCA-1, a pattern characteristic of pokeweed mitogen-stimulated B cells and IgM-secreting macroglobulinemia cells.[11,14] RPMI 8226, the only other B cell line tested that lacked HLA-DR, expressed low amounts of B1 and B2. Thus, these two cell lines appear to represent less differentiated secretory cells than NCI-H929. Some other "plasmacytoid" lines[1] have not been independently confirmed or characterized antigenically. Recent reports have suggested that only two or three human myeloma lines exist,[1,43] and the failure of any human line to emerge as a widely used fusion partner for the generation of human monoclonal antibodies[43,44] is further evidence of the dearth of true human myeloma lines.

NCI-H929 tumor and cultured cells demonstrated an identical rearrangement of the *c-myc* gene. The rearranged DNA fragment was readily observable on *Eco*RI digests of genomic DNA, and its concentration increased relative to the germ line band during culture. The use of other restriction enzyme digests excluded the possibility that the DNA rearrangement was due to a simple *Eco*RI restriction enzyme site polymorphism. The relative increase of the rearranged band during culture could be due to (a) loss of contaminating stromal cells during culture; (b) selective growth of a tumor cell subpopulation having the rearrangement; or (c) selective increase in the copy number of the rearranged gene. DI studies excluded significant contamination of the tumor preparation with stromal cells. The tumor cells consisted predominantly of near-diploid cells with a minor near-tetraploid subpopulation. During culture, selective growth of the latter subpopulation occurred. The near-tetraploid cells

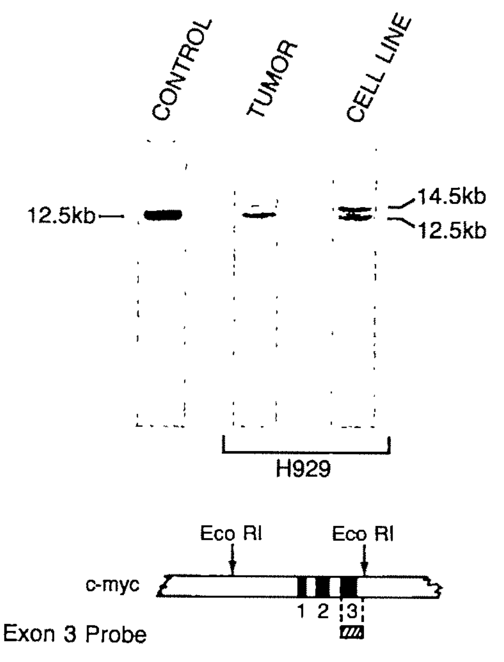

Fig 7. Genomic blot analysis for *c-myc* in NCI-H929 tumor and cell line. Three DNA samples, control, NCI-H929 tumor, and cell line are shown in an *Eco*RI Southern genomic blot analysis. The third exon of human *c-myc* probe identifies the germ line 12-kb *Eco*RI fragment in all three samples, and an additional rearranged 14.5-kb band is present in the tumor and cell line. The relative intensity of the rearranged band is greater in the cell line than in the tumor sample.

had six copies of chromosome 8, and four to six of these had an 8q+ abnormality. In BL, a reciprocal translocation occurs between the long arm of a single copy of chromosome 8 near the site of the *c-myc* gene (8q24) and one of the Ig loci (kappa, 2p11; mu, 14q32; lambda, 22q11).[3-5] In NCI-H929 cells, no structural abnormality of chromosomes 2, 14, or 22 was apparent. Of interest, in the human leukemic cell line HL-60, an amplified, overexpressed *c-myc* gene has been localized to an 8q+ abnormality.[45] At present, we are investigating the precise nature of the DNA rearrangement in NCI-H929 cells and its relationship to the 8q+ abnormality by molecular cloning. Preliminary evidence indicates that the rearrangement has taken place within or close to the third exon of *c-myc*.

There is a correlation between expression, amplification, and regulation of *c-myc* and the growth of normal and malignant cells.[46,47] Thus, our ability to readily establish a human myeloma cell culture may be related to a rearranged (and perhaps inappropriately regulated) *c-myc* gene. The culture was initiated after the patient had entered an aggressive terminal phase, characterized by rapid proliferation, massive extramedullary disease, cytologic atypia, and imminent death.[48] Whether the alterations in tumor behavior (and perhaps rearrangement of the *c-myc* gene) were associated with or induced by the therapy is unknown. However, one of the drugs she received, melphalan, induces mutations in mouse myeloma cells.[49] In addition, the use of a serum-free defined medium may have been critical for culture establishment, as the tumor cells initially failed to proliferate in serum containing media.

NCI-H929 is a highly differentiated human PC line. Antigenic and molecular studies confirm that it is derived from the patient's myeloma cells. It is an important addition to a small, select number of similar human lines, and in fact, it may be unique in the degree of its differentiation and quantity of Ig secreted. In addition to being a potential fusion partner for human monoclonal antibody production, the culture offers a model for studying terminal B cell differentiation, Ig secretion, and the relationship between human myeloma cells and the *c-myc* gene.

NOTE ADDED IN PROOF

Since acceptance of this manuscript for publication, the authors have become aware of two further reports of EBNA negative human plasmacytoid lines having varying patterns of antigen expression.[50,51] In addition, a report by Sumeji and co-workers[52] examined myeloma and PC leukemia cells for rearrangement, amplification, and expression of c-*myc*. They found no rearrangement or amplification of c-*myc* in 21 cases of myeloma. In contrast, two out of three cases of PC leukemia showed amplification of the gene with concomitant higher level of expression.

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REFERENCES

1. Karpas A, Fischer P, Swirsky D: Human myeloma cell line carrying a Philadelphia chromosome. Science 216:997, 1982

2. Nilsson K, Klein G: Phenotypic and cytogenetic characteristics of human B-lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. Adv Cancer Res 37:319, 1982

3. Marcu KB, Harris LJ, Stanton LW, Erikson J, Watt R, Croce CM: Transcriptionally active *c-myc* oncogene is contained within NIARD, a DNA sequence associated with chromosome translocations in B-cell neoplasia. Proc Natl Acad Sci USA 80:523, 1983

4. Nishikura K, Ar-Rushdi A, Erikson J, Watt R, Croce CM: Differential expression of the normal and of the translocated human *c-myc* oncogenes in B cells. Proc Natl Acad Sci USA 80:4822, 1983

5. Taub R, Moulding C, Battey J, Murphy W, Vasicek T, Lenoir GM, Leder P: Activation and somatic mutation of the translocated *c-myc* gene in Burkitt lymphoma. Cell 36:339, 1984

6. Mushinski JF, Bauer SR, Potter M, Reddy EP: Increased expression of myc-related oncogene mRNA characterizes most BALB/c plasmacytomas induced by pristane or Abelson murine leukemia virus. Proc Natl Acad Sci USA 80:1073, 1983

7. Magrath IT, Pizzo PA, Whang-Peng J, Douglass EC, Alabaster O, Gerber P, Freeman CB, Novikovs L: Characterization of lymphoma-derived cell lines positive and negative for Epstein-Barr virus nuclear antigen. J Natl Cancer Inst 64:465, 1980

8. Sumegi J, Spira J, Bazin H, Szpirer D, Levan G, Klein G: Rat c-myc oncogene is located on chromosome 7 and rearranges in immunocytomas with t(6:7) chromosomal translocation. Nature 306:497, 1983

9. Philip P: Chromosomes of monoclonal gammopathies. Cancer Genet Cytogenet 2:79, 1980

10. Wall R, Kuehl M: Biosynthesis and regulation of immunoglobulins. Ann Rev Immunol 1:393, 1983

11. Halper J, Fu SM, Wang CY, Winchester R, Kunkel HG: Patterns of expression of human Ia-like antigens during the terminal stages of B cell development. J Immunol 120:1480, 1978

12. Ritz J, Nadler LM, Bhan AK, Notis-McConarty J, Pesando JM, Schlossman SF: Expression of common acute lymphoblastic leukemia antigen (CALLA) by lymphomas of B-cell and T-cell lineage. Blood 58:648, 1981

13. Nadler LM, Ritz J, Hardy R, Pesando JM, Schlossman SF: B4, a human B lymphocyte associated antigen expressed on normal, mitogen-activated and malignant B lymphocytes. J Immunol 131:244, 1983

14. Stashenko P, Nadler LM, Hardy R, Schlossman SF: Expression of cell surface markers after human B lymphocyte activation. Proc Natl Acad Sci USA 78:3848, 1981

15. Anderson KC, Bates MP, Slaughenhoupt BL, Pinkus GS, Schlossman SF, Nadler LM: Expression of human B cell-associated antigens on leukemias and lymphomas: A model of human B cell differentiation. Blood 63:1424, 1984

16. Anderson KC, Park EK, Bates MP, Leonard RC, Hardy R, Schlossman SF, Nadler LM: Antigens on human plasma cells identified by monoclonal antibodies. J Immunol 130:1132, 1983

17. Ruiz-Arguelles GJ, Katzman JA, Greipp PR, Gonchoroff NJ, Garton JP, Kyle RA: Multiple myeloma: Circulating lymphocytes that express plasma cell antigens. Blood 64:352, 1984

18. Latreille J, Barlogie B, Johnston D, Drewinko B, Alexanian R: Ploidy and proliferative characteristics in monoclonal gammopathies. Blood 59:43, 1982

19. Gazdar AF, Oie HB: Cell culture methods for human lung cancer. Cancer Genet Cytogenet 19:5, 1986

20. Krishan A: Rapid cytofluorometric analysis of mammalian cell cycle by propidum iodine staining. J Cell Biol 66:188, 1975

21. Joliff CR, Cost KM, Stivirins PC, Grossman PP, Nolte CR, Franco SM, Fijan KJ, Fletcher LL, Shriner HC: Reference intervals for serum IgG, IgA, C3 and C4 as determined by rate nephelometry. Clin Chem 28:126, 1982

22. Nilsson K, Bennich H, Johansson SG, Ponten J: Established immunoglobulin producing myeloma (IgE) and lymphoblastoid (IgG) cell lines from an IgG myeloma patient. Clin Exp Immunol 7:477, 1970

23. Matsuoka Y, Moore GE, Yagi Y, Pressman D: Production of free light chains of immunoglobulin by a hematopoietic cell line derived from a patient with multiple myeloma. Biol Med 125:1246, 1967

24. Schwaber HS, Posner MR, Schlossman SF, Lazarus H: Human-human hybrids secreting pneumococcal antibodies. Hum Immunol 9:137, 1984

25. Burk KH, Drewinko B, Trujillo JM, Ahearn MJ: Establishment of a human plasma cell line in vitro. Cancer Res 38:2508, 1978

26. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor, NY, CSH Press, 1982, p 196

27. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

28. Chirgwin J, Przybyla A, McDonald R, Rutter W: Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 18:5294, 1979

29. Ravetch JV, Kirsch IR, Leder P: Evolutionary approach to the question of immunoglobulin heavy chain switching: Evidence from cloned human and mouse genes. Proc Natl Acad Sci USA 77:6735, 1980

30. Hieter PA, Max EE, Seidman JG, Maizel JV, Leder P: Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 22:197, 1980

31. Battey J, Moulding C, Taub R, Murphy W, Steward T, Potter H, Lenoir G, Leder P: The human c-myc oncogene: Structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 34:779, 1983

32. Hayhoe FG, Quaglino D: Haematological Cytochemistry New York, Churchill Livingstone, 1980, p 50

33. Arnold A, Grossman J, Bakhshi A, Jaffee ES, Waldman TA, Korsmeyer SJ: Immunoglobulin gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J Med 309:1593, 1983

34. Benjamin D, Magrath IT, Maguire R, Janus C, Todd HD, Parsons RG: Immunoglobulin secretion by cell lines derived from African and American undifferentiated lymphomas of Burkitt's and non-Burkitt's type. J Immunol 129:1336, 1982

35. Fingeroth JD, Weiss JJ, Tedder TF, Strominger JL, Biro PA, Fearon DT: Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc Natl Acad Sci USA 81:4510, 1984

36. Trowbridge IS, Hopez F: Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro. Proc Natl Acad Sci USA 78:4515, 1981

37. Van Driel IR, Stearne PA, Greco B, Simpson RJ, Goding JW: The receptor for transferrin on murine myeloma cells: One step purification based on its physiology, and partial amino acid sequence. J Immunol 133:3220, 1984

38. Diehl V, Schaadt M, Kirchner H, Hellriegel UP, Gudat F, Fonatsch C, Laskewitz E, Guggenhein R: Long term cultivation of plasma cell leukemia cells and autologous lymphoblasts in vitro: A comparative study. Blut 36:331, 1978

39. Ritts RE, Ruiz-Arguelles JR, Wehl KG, Sundt TM, Bradley AL, Leduc PV, Kyle RA: Establishment and characteristics of a human non secretory plasmacytoid cell line. Immunobiology 160:95, 1981

40. Miller CH, Carbonell A, Peng MS, Paglieroni T, MacKensie MR: A human plasma cell line in vitro. Cancer 49:2091, 1982

41. Croce CM, Shander M, Martinis J, Cicurel L, D'Ancona GG, Dolby TW, Koprowski H: Chromosomal location of the genes for human immunoglobulin heavy chains. Proc Natl Acad Sci USA 76:3416, 1979

42. Sikora K, Alderson T, Phillips J, Watson JV: Human hybridomas from malignant gliomas. Lancet 1:11, 1982

43. Kozbor D, Dexter D, Roder JC: A comparative analysis of the phenotypic characteristics of available fusion partners for the construction of human hybridomas. Hybridoma 2:7, 1983

44. Massicotte H, Rauch J, Stoenfeld Y, Tannenbaum H: Influence of fusion cell plating density on production of human-human hybridomas secreting anti-DNA autoantibodies from patients with systemic lupus erythematosus. Hybridoma 3:215, 1984

45. Nowell P, Finan J, Dalla-Favera R, Gallo RC, ar-Rushdi A, Romanczuk H, Selden JR, Emmanuel BS, Rovera G, Croce CM: Association of amplified oncogene *c-myc* with an abnormally banded chromosome 8 in a human leukemia cell line. Nature 306:494, 1983

46. Kelly K, Siebenlist U: The role of c-myc in the proliferation of normal and neoplastic cells. J Clin Immunol 5:65, 1985

47. Gazdar AF, Carney DN, Nau MM, Minna JD: Characterization of variant subclasses of cell lines derived from small cell lung cancer having distinctive biochemical, morphological and growth properties. Cancer Res 45:2924, 1985

48. Suchman AL, Coleman M, Mouradian JA, Wolf DJ, Saletan S: Aggressive plasma cell myeloma: A terminal phase. Arch Intern Med 141:1315, 1981

49. Preud'homme JL, Buxbaum J, Schraff MD: Mutagenesis of mouse myeloma cells with melphalan. Nature 245:320, 1973

50. Katagiri S, Yonezawa T, Kuyama J, Kanayama Y, Nishida K, Abe T, Tamaki T, Ohnishi M, Tarui S: Two distinct human myeloma cell lines originating from one patient with myeloma. Int J Cancer 36:241, 1985

51. Durie GM, Vela E, Baum V, Leibovitz A, Payne CM, Richter LC, Grogan TM, Trent JM: Establishment of two new myeloma cell lines from bilateral pleural effusions: Evidence for sequential in vivo clonal change. Blood 66:548, 1985

52. Sumegi J, Hedberg T, Bjorkholm M, Godal T, Mellstedt H, Nilson MG, Perlman C, Klein G: Amplification of the c-*myc* oncogene in human plasma-cell leukemia. Int J Cancer 36:367, 1985.

Purification of Hageman Factor (Factor XII) on Columns of Popcorn-Agarose

By Oscar D. Ratnoff, Barbara Everson, Virginia H. Donaldson and Bridget H. Mitchell

Purification of Hageman factor (HF, factor XII) from human plasma is a tedious procedure and the product is not always in the precursor form. Hojima has described a protein derived from corn kernels that inhibits the enzymatic properties of HF. This inhibitor binds to the precursor form of HF. Rapid purification of HF was achieved by using as the major purification step adsorption of this clotting factor to popcorn inhibitor bound to agarose. The product had a specific activity of 50.0 to 67.1 coagulant units of HF per milligram protein, and the yield was 33% to 40% of the HF content of the starting plasma. The purified protein displayed a single band upon unreduced or reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis and less than 0.1% was in an activated form, as measured in coagulant assays. The technique described is more rapid and reliable than methods described earlier.

WE REPORTED RECENTLY that Hageman factor (HF, factor XII) in its precursor state is readily complexed to popcorn inhibitor that has been bound to cyanogen bromide agarose, and that HF can then be eluted from this matrix.[1] This procedure has allowed us to develop a simplified method for the purification of HF that is essentially uncontaminated with activated forms of this clotting factor. This has made it possible to purify HF more readily and more reliably than by earlier methods, and appears to avoid contamination with activated species of HF that are sometimes found in preparations separated by other techniques.[2,3]

MATERIALS AND METHODS

After obtaining informed consent as approved by our institutional committee on the protection of the rights of human subjects, blood for purification of HF was drawn from the antecubital veins of volunteer donors into one-fiftieth volume of 0.5 mol/L sodium citrate buffer (pH 5.0) containing 31 mg/mL of benzamidine hydrochloride (Sigma Chemical Co, St Louis, Mo) and 10 mg/mL of hexadimethrine bromide (Polybrene, Sigma). The blood was centrifuged at 2 °C for 20 minutes at 12,000 g in 250 mL polycarbonate bottles (Du Pont Co, Wilmington, Del). The plasma was withdrawn with polystyrene pipettes, and the plasma recentrifuged in the same manner. Blood from patients with Hageman trait, classic hemophilia (factor VIII "deficiency") or parahemophilia (deficiency of proaccelerin or factor V) was withdrawn into the same anticoagulant without benzamidine or hexadimethrine bromide and plasma was separated in the same way. Human plasmas deficient in prothrombin, factor VII, Christmas factor (factor IX), Stuart factor (factor X), high molecular weight kininogen (Fitzgerald factor) or plasma prekallikrein (Fletcher factor) were purchased from George King, Overland Park, Kansas. Bovine plasma deficient in plasma thromboplastin antecedent (PTA, factor XI) was the gift of Dr. Patricia Gentry, Guelph, Ontario. Pooled normal plasma, used as a standard, was prepared from the venous blood of 24 normal men aged 21 to 40 and stored at −70 °C as described earlier.[4] This pool was arbitrarily defined as containing 1.00 U/ml of each clotting factor tested.

Popcorn-agarose HF inhibitor was prepared and, after use, regenerated as previously described.[1] Other reagents used included lysine agarose,[4] diethyl(2-hydroxypropyl)aminoethyl Sephadex A-50 (QAE-Sephadex, Pharmacia Fine Chemicals, Piscataway, NJ), sulfopropyl Sephadex C-50 (SP-Sephadex, Pharmacia), bovine albumin (crystallized, Pentex, Miles Laboratories, Elkhart, Ind), ammonium acetate (Sigma), ammonium sulfate (ultrapure, Schwarz-Mann, Cambridge, Mass), hexadimethrine bromide (Polybrene, Sigma, St Louis), benzamidine hydrochloride (Sigma), tissue thromboplastin (rabbit brain, Sigma, suspended in accordance with the manufacturer's directions), soy bean phosphatides (Centrolex "P", the gift of Central Soya Co, Fort Wayne, Ind), kaolin (acid-washed, NF, Fisher Scientific Co, Fairlawn, NJ), streptokinase (Streptase, Behringwerke AG, Marburg, W. Germany), H-D-valyl-leucyl-lysine *p*-nitroanilide hydrochloride (S2251, Helena Laboratories, Beaumont, Tex), and H-D-prolyl-L-phenylalanyl-L-arginine *p*-nitroanilide (S2302, Helena).

The buffers used included sodium phosphate buffer (0.1 mol/L, pH 7.5), barbital-saline buffer (0.025 mol/L sodium barbital, 0.125 mol/L sodium chloride, pH 7.5), barbital-saline-albumin buffer (barbital-saline buffer containing 1% bovine albumin), barbital-saline-Polybrene buffer (barbital-saline buffer containing 100 mg/L hexadimethrine bromide), Tris buffer [0.025 mol/L tris(hydroxymethyl)aminomethane, 0.002 mol/L benzamidine hydrochloride, 50 mg/L hexadimethrine bromide, 0.1 mmol/L disodium ethylenediamine tetraacetic acid, 0.1 mol/L sodium chloride, and enough 4N hydrochloric acid to bring the pH to 8.0], acetate buffer (0.05 mol/L sodium acetate, 0.15 mol/L sodium chloride, pH 5.2), acetate-Polybrene buffer (acetate buffer containing 100 mg/L hexadimethrine bromide), and ammonium acetate–sodium chloride buffer (0.1 mol/L ammonium acetate in 1.0 mol/L sodium chloride and 100 mg/L hexadimethrine bromide, pH 6.8). The ammonium acetate–sodium chloride buffer was prepared freshly for each experiment. All buffers used for the preparation of HF contained 0.02% sodium azide.

Except as noted, all glassware was rinsed with dichlorooctamethyltetrasiloxane (SurfaSil, Pierce Chemical Co, Rockford, Ill) and dried overnight at room temperature. The Büchner funnels used were silicone-coated in the same way. All dialyses were carried out in SpectraPor cellulose casings (mol wt cutoff 12,000 to 14,000 kD, Spectrum Medical, Los Angeles, Calif). All centrifugations were at 4 °C, those at 12,000 g in a Sorvall RC-2 centrifuge (Dupont Co, Newton, Conn), and those at 800 g in an International PR-2 centrifuge (Damon/International Equipment Co, Needham, Mass).

To purify HF, the plasma was first depleted of plasminogen by mixing at room temperature in a polycarbonate beaker approximately 300 mL of citrated plasma, prepared as described above and diluted with an equal volume of distilled water, with 150 mL settled volume of a suspension of lysine-agarose that had been equilibrated

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in 0.1 mol/L sodium phosphate buffer (pH 7.5). The mixture was stirred gently for 30 minutes and allowed to stand for an additional hour. It was then filtered through a Büchner funnel fitted with Whatman No. 541 filter paper and the plasminogen-depleted plasma was collected by suction into an Erlenmeyer flask. The gel was washed with approximately 50 mL of Tris buffer, combining the filtrate with the filtered plasma. The filtrate was then dialyzed overnight at room temperature against two changes of 6 L of Tris buffer (pH 8.0).

The dialyzed plasma was transferred to a polycarbonate beaker and mixed at room temperature for one hour with 500 mL settled volume of QAE-Sephadex gel that had been equilibrated with Tris buffer. The mixture was allowed to stand at room temperature for 30 additional minutes and was then filtered by suction through a Büchner funnel lined with Whatman No. 541 filter paper. The QAE-Sephadex gel, to which HF was adsorbed, was washed with 5 L of Tris buffer. The gel was transferred to a polycarbonate beaker and mixed with 150 mL of Tris buffer containing 0.5 mol/L sodium chloride. The mixture was refiltered through a Büchner funnel as before, collecting the filtrate in an Erlenmeyer flask and saving the filtrate. The gel was again transferred to a polycarbonate beaker, mixed with Tris buffer containing 0.5 mol/L sodium chloride and refiltered, a process repeated two additional times, which was sufficient to elute the bulk of HF from the QAE-Sephadex gel. The four filtrates were combined, mixed at 4 °C with 390 g/L of solid ammonium sulfate (ie, to 60% saturation), and the precipitate that formed was allowed to settle overnight.

The mixture was centrifuged at 12,500 g for 20 minutes at 4 °C and the supernatant fluid decanted. The precipitate was dissolved in as little Tris buffer as possible and dialyzed against running tap water for three hours and against Tris buffer at 4 °C overnight. The dialyzed sample, approximately 50 to 100 mL, was centrifuged at 900 g for ten minutes at 2 °C to remove undissolved matter. The supernatant fraction was then applied to a 15- to 25-mL (packed volume) column of popcorn-agarose, equilibrated with barbital-saline-Polybrene buffer, in a 60-mL polypropylene syringe, supported by nylon mesh that had been rinsed in hexadimethrine bromide (10 mg/mL water). The column was washed with 20 mL of the same buffer, and then eluted with ammonium acetate–sodium chloride buffer (pH 6.8), collecting 1.5-mL fractions in polystyrene tubes at 4 °C overnight. Tubes containing HF were identified by coagulant assays. The approximately 50 to 80 mL of eluate that had appreciable HF coagulant properties was stored in polypropylene tubes at −70 °C until used.

The eluate was dialyzed overnight at 4 °C against acetate-Polybrene buffer and applied at the same temperature to a 1.5 × 30-cm column of SP-Sephadex that had been equilibrated in the same buffer. The column was eluted with a linear gradient of 200 mL of acetate buffer in the proximal chamber and 200 mL of the same buffer containing 0.5 mol/L sodium chloride in the distal chamber, collecting 2-mL fractions in polystyrene tubes. Neither of these buffers contained hexadimethrine bromide. Tubes containing HF were identified by coagulant assays. Those fractions with the highest activity, approximately 40 mL, were pooled and stored at −70 °C in small aliquots in polypropylene containers.

Coagulant assays of clotting factors were performed in uncoated glass tubes by modifications of the activated partial thromboplastin time and prothrombin time referred to earlier.[5] All samples were diluted in barbital-saline-albumin buffer to provide stability during the assays. Coagulant assays for activated HF were performed by the same technique, using polystyrene tubes (internal diameter 10 mm) coated with SurfaSil and omitting kaolin from the kaolin-phospholipid mixture. Total and activated HF were also assayed by an amidolytic method, as previously reported,[2] using a concentration of HF of 0.004 coagulant units per mL in the enzyme-substrate mixture. Plasminogen was assayed by amidolysis of H-D-valyl-L-leucine-*p*-nitroanilide dihydrochloride (S2251), as described earlier.[5]

The protein concentration at each step was measured by Lowry's method.[6]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of reduced and unreduced samples of purified HF was performed as described earlier and calibrated against a mixture of proteins with defined molecular weights (Electrophoresis Calibration Kit, Pharmacia).[7]

RESULTS

Batch adsorption with QAE-Sephadex gel and subsequent precipitation with ammonium sulfate reduced the protein content of plasminogen-depleted plasma to about one fifth to one seventh that of the original plasma with about a 40% loss of HF titer (Table 1). Subsequent filtration of the partially purified HF through popcorn-agarose gel resulted in a further loss of about 30% of the HF content of the original plasma, an estimate that may have been exaggerated by the effect of inhibitors in the eluting buffer upon the coagulation assay; there was an increase in the specific activity to about 1,300-fold compared to the starting plasma (Fig 1). This preparation was contaminated with a high molecular weight species of protein that could not be separated readily on Sephadex gels. It was therefore chromatographed upon SP-Sephadex gel (Fig 2) without appreciable loss of HF titer; the eluted HF had been purified about 5,000-fold over the starting plasma and in different preparations had a specific activity of 50.0 to 67.1 U/mg protein with yields of

Table 1. Purification of Hageman Factor

Sample	Volume (mL)	Activity (U/mL)	Total Units	Protein (mg/mL)	Total Protein (mg)	Specific Activity (U/mg Protein)	Yield (%)
Plasma dil 50%	600	0.42	252	32.0	19,200	0.013	100
Lys agar ads plasma	660	0.40	264	24.0	15,840	0.017	100
Dial amm sulf ppt	90	1.76	158	39.5	3,555	0.045	63
PCI eluate	60	1.42	85*	0.109	6.54	13.0	37*
SP Seph pool	39	2.56	100	0.044	1.72	58.2	40

Abbreviations: Lys agar plasma, lysine-agarose-adsorbed, diluted plasma; Dial amm sulf ppt, ammonium sulfate precipitate of lys agar plasma after adsorption to and elution from QAE-Sephadex after dialysis against Tris buffer; PCI eluate, popcorn inhibitor–agarose eluate of ammonium sulfate precipitate after dialysis; SP Seph pool, pooled eluates of PCI eluate adsorbed to SP-Sephadex gel.

*This may have been an underestimate because of the presence of inhibitors in the buffer.

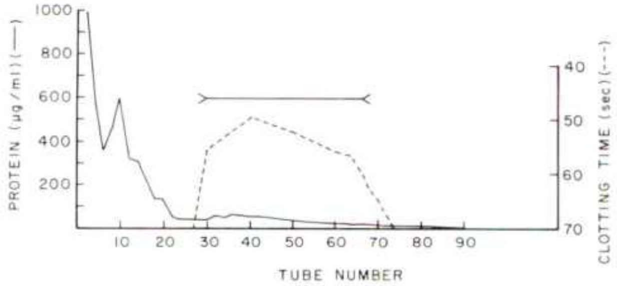

Fig 1. Filtration of partially purified HF through popcorn-agarose gel. 58 mL of ammonium sulfate-precipitated fraction (114 U HF) was applied to a 22-ml column of popcorn agarose equilibrated with barbital-saline-Polybrene buffer. Elution with 0.1 mol/L ammonium acetate buffer (pH 6.8) containing 1.0 mol/L sodium chloride and 100 mg/L hexadimethrine bromide was begun at Tube 1 of the Figure, collecting 1.5-mL fractions. The titer of HF, estimated by specific coagulant assay, is represented in terms of clotting times. The contents of tubes 30 through 66 were pooled (>—<).

33% to 40% of the HF content of the starting plasma. It was devoid of other clotting factors and plasminogen (Table 2). The HF was less than 0.1% in the activated form in coagulant assays, and had no detectable amidolytic properties unless activated by ellagic acid (Table 3). Usually a single band was visible upon SDS-PAGE of reduced and unreduced HF; the molecular weight appeared to be approximately 75,000 kD in comparison to a series of protein standards (Fig 3). In one preparation, the protein band appeared to be a doublet, as was occasionally observed in preparations made by earlier methods. Preparation of HF by the method described required about ten working days. The coagulant

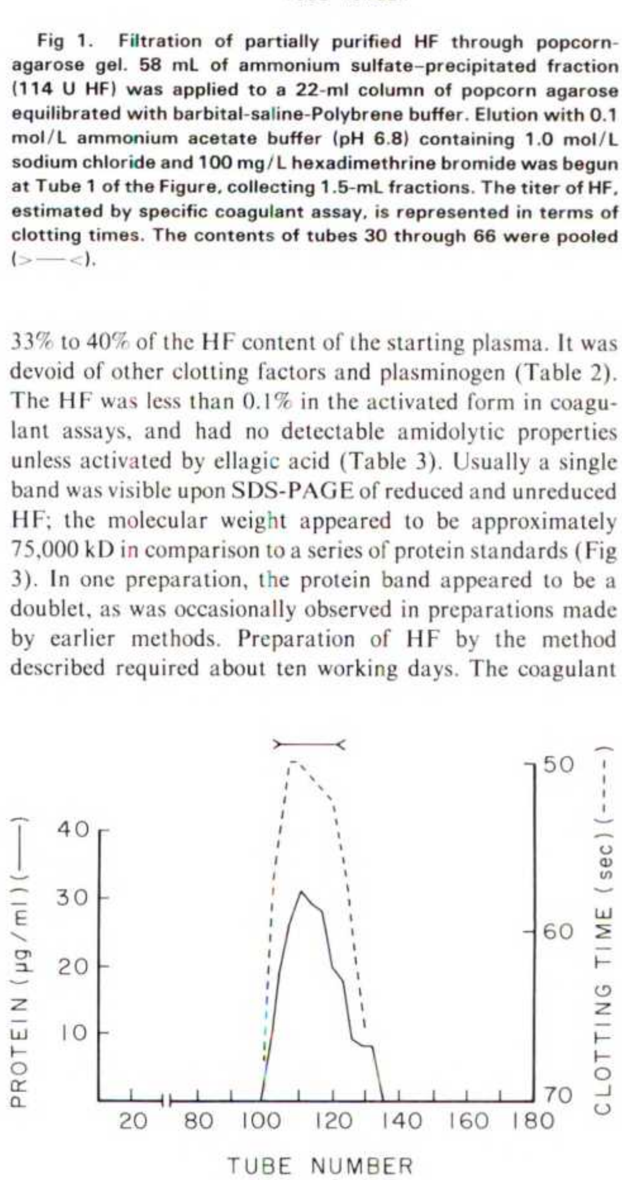

Fig 2. Chromatography of partially purified HF through SP-Sephadex gel. 76 mL of popcorn-agarose eluate (76 U HF) was applied to a 1.5 × 30-cm column of SP Sephadex gel equilibrated with 0.05 mol/L sodium acetate buffer (pH 5.2) in 0.15 mol/L sodium chloride. A linear gradient of this buffer and the same buffer containing 0.50 mol/L sodium chloride was begun at Tube 1 of the Figure, collecting 2.0-mL fractions. The titer of HF, estimated by specific coagulant assay, is represented in terms of clotting times. The contents of tubes 104 through 122 were pooled (>—<).

Table 2. Assays of Purified Hageman Factor for Clotting Factors and Plasminogen

Factor	Pooled Plasma* Clotting Time (sec)	Purified HF† Clotting Time (sec)	Purified HF† U/mL
Prothrombin	33.4	35.0	<0.005
Proaccelerin (factor V)	45.6	47.8	<0.005
Factor VII	37.2	39.0	<0.005
AHF (factor VIII:C)	95.8	101.4	<0.005
Christmas factor (factor IX)	105.9	107.6	<0.005
Stuart factor (factor X)	113.8	140.2	<0.005
PTA (factor XI)	125.1	176.7	<0.005
HMW kininogen	232.3	392.1	<0.005
Plasma prekallikrein	194.7	207.3	<0.005
	Percent‡	Percent‡	
Plasminogen	100	<0.04	

*Clotting time of pooled normal plasma, diluted 2,000-fold, in specific assays for the listed clotting factors.

†Clotting time of purified HF (2.6 U/mL), diluted 20-fold, in specific assays for the listed clotting factors.

‡Amidolytic activity of streptokinase-activated plasma (diluted 100-fold) compared to that of purified HF, tested undiluted.

Table 3. Spontaneous Amidolytic Activity of Purified Hageman Factor

Test Mixture	Amidolytic Activity nmol *p*NA/mL/60 min
HF + ellagic acid	26.9
HF + buffer	0.0

Amidolytic activity of a mixture of 0.05 mL HF (0.05 U/mL 0.25% bovine albumin in barbital-saline buffer, 0.1 mL Dulbecco's phosphate-buffered saline and 0.05 mL 2×10^{-5} mol/L ellagic acid in barbital-saline buffer, incubated at 37 °C in 10 × 75-mm polystyrene tubes for 60 min, after which 1.0 mL 5×10^{-4} mol/L S2302 was added and incubation was continued for 60 min. The reaction was stopped and the amount of *p*-nitroaniline (*p*NA) released was measured spectrophotometrically at 405 nm.

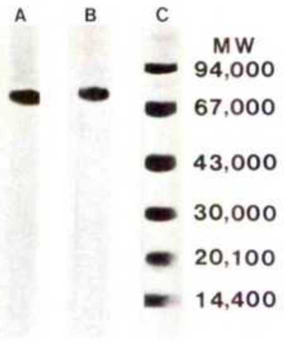

Fig 3. SDS-PAGE of purified HF. A volume of 50 μL HF (310 μg/mL, specific activity 67 U/mg protein) was applied to (A) and (B); the HF in (A) was unreduced, and that in (B) was reduced. Protein markers (C), whose mol wts are depicted on the right, were tested after reduction.

HF titer was unaltered by storage in polypropylene containers at −70 °C for 30 days.

DISCUSSION

Hojima et al[8] reported that a protein derived from sweet corn, apparently identical to one known to inhibit trypsin, inhibited activated HF. This agent, derived at Hojima's suggestion from popcorn, appeared to inhibit the activation of HF from its precursor.[1] Kambhu et al[1] demonstrated that the popcorn inhibitor, rendered insoluble by binding to cyanogen bromide–agarose gels, adsorbed the precursor form of HF from plasma. The HF could be eluted gradually from the gel by washing with barbital-saline buffer, and much more efficiently by the same buffer containing 1.0 mol/L sodium chloride. The preparations obtained, however, had low specific activity and were unsuitable for experimental use.

The technique described in the present report took advantage of this unusual property of the popcorn inhibitor, and made possible a major simplification of the procedure for purification of HF. Speed was also achieved by initial purification of HF by batch elution of plasminogen-depleted plasma on QAE Sephadex. The eluate was further purified by chromatography on popcorn-agarose, but was still contaminated with a high molecular weight protein that was readily removed by SP-Sephadex chromatography. The final product was essentially all in the precursor form, as assessed by coagulant and amidolytic assays.

The procedure described, then, allowed much more rapid purification of HF than did earlier methods, and the product was more reliably obtained in the precursor form.

REFERENCES

1. Kambhu SA, Ratnoff OD, Everson BA: Inhibition of Hageman factor (factor XII) by popcorn inhibitor. J Lab Clin Med 105:625, 1985

2. Ratnoff OD, Saito H: The evolution of clot-promoting and amidolytic activities in mixtures of Hageman factor (factor XII) and ellagic acid. J Lab Clin Med 100:248, 1982

3. McMullen BA, Fujikawa K: Amino acid sequence of the heavy chain of human α-factor XIIa (activated Hageman factor). J Biol Chem 260:5328, 1985

4. Zimmerman TS, Ratnoff OD, Powell AE: Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease with observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J Clin Invest 50:244, 1971

5. Ratnoff OD, Nossel HL: Wasp sting anaphylaxis. Blood 61:132, 1983

6. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

7. Weber K, Osborn M: The reliability of molecular weight determination by dodecyl sulfate–polyacrylamide gel electrophoresis. J Biol Chem 244:4406, 1969

8. Hojima Y, Pierce JV, Pisano JJ: Plant inhibitors of serine proteinases: Hageman factor fragment, kallikreins, plasmin, thrombin, factor Xa, trypsin and chymotrypsin. Thromb Res 20:163, 1980

Carrier Detection in Hemophilia A: A Cooperative International Study.
I. The Carrier Phenotype

By John B. Graham, Charles R. Rizza, Juan Chediak, Piero Mannuccio Mannucci, Ernest Briët, Rolf Ljung, Carol K. Kasper, E.M. Essien, and P.P. Green

Eight laboratories in six countries cooperated to clarify several issues concerning the phenotypes of heterozygous carriers of hemophilia "A." Plasma levels of factor VIII (F.VIII:C, formerly VIII:C) and von Willebrand factor (VWF:Ag, formerly VIIIR:Ag) of carriers and normal women were determined by various "in-house" methods; a single lyophilized plasma standard was used for all assays. Analysis of the collated data from 336 carriers (296 obligatory carriers and 40 sporadic carriers) and 137 normal women showed that there was no difference in the F.VIII:C levels of "paternal" carriers (women who had obtained the abnormal gene from their fathers) and "maternal" carriers. Neither was there a difference in the VWF:Ag levels of normal women and either type of carrier. Age was found to have a significant effect on both F.VIII:C and VWF:Ag, values being higher at very young and very old ages, the minima occurring in the 25- to 30-year range. ABO blood type had a striking effect. Women of types A, B, and AB (designated non-O in the study), both normals and carriers, had significantly higher levels of both factors than did women of type O. Analysis by laboratories showed that differences in mean levels of both factors between laboratories were highly significant. It was concluded that age, ABO blood type, and laboratory variation should be taken into account in carrier detection.

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IDENTIFICATION of heterozygotes for hemophilia "A" (detection of carriers) by laboratory means was first attempted by Merskey and Macfarlane in 1951,[1] prior to the introduction of factor VIII (F.VIII) assays for this purpose by Graham and colleagues[2] in 1953. Veltkamp and coworkers applied linear discriminant analysis to factor VIII assays in 1968,[3] and Zimmerman and colleagues[4] showed in 1971 that detection could be improved by using assays for F.VIII together with assays for an antigen missing in severe von Willebrand's disease (VWD).*

Linear discriminant analysis was applied to conjoint F.VIII:C and VWF:Ag assays by Elston and colleagues in 1976.[5] Reisner and colleagues[6] extended the analysis to three variables by measuring in addition the rate of agglutination of platelets in the presence of ristocetin. Multivariate analysis has been applied to carrier detection by Wahlberg and co-workers[7] who used all of the appropriate plasma variables plus screening tests and pedigree data.

It is not widely appreciated that all available information should be used when the result of a diagnostic procedure, such as phenotyping hemophilia carriers, is a probability. Four kinds of information are sought: (a) information about the antecedents of a possible carrier and transmission of the trait through her family; (b) information about her descendants; (c) information about linkage of the hemophilia gene to other X-linked traits in her family, eg, colorblindness or G6PD types, etc.; and (d) bioassays of her plasma and that of her relatives. The first three are combined mathematically to provide a prior probability, and the fourth provides the likelihood ratio. The final probability is produced by combining these. It is essential that at least one affected male in each kindred be definitively studied to assure that a correct diagnosis has been made and to establish the degree of severity, principles which have been discussed at length elsewhere.[8-11] The latest development has been the genotyping of subjects using restriction fragment-length polymorphisms (RFLPS) to characterize X-chromosomal DNA, the advantages and disadvantages of which are discussed elsewhere.[12]

There have been conflicting reports concerning the effects of age and other variables on the levels of F.VIII:C and VWF:Ag in hemophilia carriers. Some workers have found that age affects F.VIII:C levels,[5,13,14] whereas others have not.[15-17] It has been reported that the F.VIII:C levels of carriers who have received the hemophilia gene from their fathers (paternal carriers) are lower than those of carriers who have received the gene from their mothers (maternal carriers),[18,19] an observation which has been disputed.[7,20-23] A differential effect between types of carriers would not be trivial, either theoretically or practically. It might be a signal that paternally and maternally derived X-chromosomes have different probabilities of inactivation, which would require that a reference group of carriers for discriminant analysis consist solely of maternal carriers.

Another variable which might affect carrier detection is

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*The International Committee on Thrombosis and Haemostasis has recommended that the term F.VIII be used when referring generally to this clotting factor, F.VIII:C when referring to its activity, and F.VIII:Ag (formerly F.VIII:Cag) when it is measured immunologically. It has also recommended that VWF be used when referring generally to the clotting factor missing in severe VWD, and VWF:Ag (formerly VIIIR:Ag) when this factor is measured immunologically.

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Table 1. Percentage of Sample In Each Decade

Decade	0–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	Total
Carriers (N = 332)	3.3	12.6	14.2	22.3	18.7	18.1	7.5	2.7	0.6	100
Normal subjects (N = 136)	0.7	3.7	24.3	27.2	21.3	16.2	5.1	1.5	0.0	100

the effect of ABO blood type on factor levels. Levels of F.VIII:C and VWF:Ag appear to be lower in persons of type O than those of types A, B, and AB. This was first reported by Preston and Barr in 1964[15] and has been confirmed several times since.[7,13,14,24–26]

A possible explanation for the conflicting reports is that they have been based upon studies of small, sometimes nonrepresentative populations. This possibility, particularly as it has concerned the differences between types of carriers, prompted the present study. The phenotypic characteristics of hemophilia A carriers are described in this article, and an analysis of the use of linear discriminants for carrier detection comprises the accompanying report.[27]

METHODS AND MATERIALS

Experimental design. Each laboratory obtained blood from members of its group of carriers in its particular way. Plasma was prepared and assays were performed in each laboratory by current "in-house" methods. All test samples were collected and assayed between June 1982 and July 1983, and the results were sent to Chapel Hill for analysis. ABO blood types were determined later.

Participating laboratories. Participating laboratories were: (a) the Haemophilia Centre, Oxford, England; (b) the University of North Carolina; (c) the Orthopaedic Hospital of the University of Southern California, Los Angeles; (d) the University Hospital, University of Lund, Malmo, Sweden; (e) the A. Bianchi Bonomi Haemophilia and Thrombosis Centre, University of Milan, Italy; (f) the University of Leiden, Netherlands; (g) the Michael Reese Hospital, University of Chicago, Chicago, Illinois; and (h) the University of Ibadan, Nigeria.

Standard control plasma. Standard control plasma was produced at the Haemophilia Centre in Oxford by pooling six normal plasmas that had been prepared by centrifugation of blood collected into a buffered citrate anticoagulant (3.8% sodium citrate in 7% HEPES buffer at pH 7.4). Nine parts of blood had been added to one part of anticoagulant. The pooled plasma was freeze-dried in 1-mL aliquots and was stored until use in each laboratory at a temperature below −30 °C. It was reconstituted in 1 mL of distilled water. Potency of the reconstituted plasma was maintained for up to two hours at room temperature, and the laboratories were asked not to refreeze and reuse the standard. The potency of the standard was determined to be 0.53 IU of F.VIII:C per ampoule by calibration against the international standard.† The VWF:Ag of the lyophilized standard plasma was arbitrarily defined as 100 U per deciliter, since an international standard was not available at the onset of the study.

Blood collection and plasma preparation. Blood was carefully collected in each laboratory to assure that all samples were handled in the same fashion; samples were centrifuged for at least 20 minutes at speeds > 3,000 rpm or forces > 2,000 g, often at room temperature. Some laboratories assayed fresh plasmas; others examined frozen and stored plasmas.

Assays. Seven laboratories performed F.VIII:C assays by a one-stage method; a two-stage procedure was used at Oxford. No differences were observed in the distributions of normal values attributable to the type of assay for F.VIII. In four laboratories, F.VIII:Ag was determined by immunoradiometric assays (IRMAs) using polyclonal human antibodies. Assays for VWF:Ag were done in all laboratories by the local adaptation of the electroimmunoassay (EIA) described originally by Laurell.[28] VWF:Ag was also determined by IRMAs in three laboratories. Heterologous antibodies were used in the EIAs, and both polyclonal and monoclonal antibodies against VWF:Ag were used in the IRMAs. VWF:Ag was also measured by a commercial enzyme-linked immunosorbent assay (ELISA) in Milan.

Population studied. Although the subjects were predominantly of west European ancestry, some subjects of African and Oriental ethnicity were introduced through the American and Nigerian subsamples. Women from a broad age range were examined to assess the effects of age on the variables. Five laboratories collected samples from normal women, and several laboratories collected samples from sporadic carriers. In one laboratory, each carrier was matched by a normal woman of the same age, whereas in four other laboratories a smaller sample of normal women was collected that covered the same age range as the carriers. Table 1 shows the study sample stratified for age and genotype.

Blood typing. ABO blood types were determined on as many of the subjects as possible. Unfortunately, it was impossible to ascertain the blood types of the subjects at four laboratories.

Criteria for inclusion in the study. Only carriers of severe hemophilia were accepted into the study, ie, women having a male relative with < 1% F.VIII:C. Each woman was classified as either a "paternal carrier," a "maternal carrier," a "sporadic carrier," or "normal," according to the following definitions: (a) *paternal carrier:* A woman whose putative father had been definitely established as having hemophilia A; (b) *maternal carrier:* A woman whose putative father was normal and who had produced hemophilic male(s) and/or carrier female(s) and was in the main line of descent of a hemophilia A pedigree; (c) *sporadic carrier:* A woman whose putative father was normal and who has had at least two hemophilic sons, or a hemophilic son and a carrier daughter, or two carrier daughters but no other hemophilic male relatives; or (d) *normal:* A woman with no hemophilic relatives.

Statistical treatment. To render laboratories comparable for the pertinent analyses and to assess interlaboratory variation, only the first assay result was used in cases in which multiple assays were performed. (A "second" assay was one based upon a separate aliquot from a single plasma sample or on a second sample.) The F.VIII:C and VWF:Ag results were logarithmically transformed to reduce skewness prior to performing the statistical tests. Regressions and tests for the effects of age, blood type, laboratory, and carrier status were performed using the General Linear Models Procedure in SAS language on an IBM 3081 computer.

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†The International Unit (IU) of F.VIII:C at the time of the study was based on a concentrate ~1.2 times the average unit of most carefully collected plasma pools. The values observed in this study were adjusted by multiplying them by 0.64 (1.2 × 0.53) to produce figures similar to those which are usually obtained in laboratories which use pooled normal plasma as the working standard.

RESULTS

The distributions by laboratory of normal women, paternal carriers, maternal carriers, and sporadic carriers are shown in Table 2. Approximately equal numbers of normal subjects, paternal carriers, and maternal carriers were examined.

F.VIII:C levels. The data obtained by comparison with the lyophilized standard and adjusted as described in the Methods section are shown in Table 3. The mean level of F.VIII:C in 137 normal women (column 1) was 106 U per deciliter; the means of the different laboratories ranged from 84 to 117. The differences of the means of normal women between laboratories was significant ($P < .01$).

The averages of the means for the paternal and maternal carriers, not adjusted for age, (columns 2 and 3) were the same, 54 U per deciliter. When adjusted to age 25, using the regression curves described below, the means were 48.0 (paternal) and 45.0 (maternal) U per deciliter. When the data were adjusted for the effects of both age and institution there was not a significant difference between types of carriers ($P = .29$). Because there was not a difference between the F.VIII:Cs of the different types of carriers, all the carrier data, including the sporadic carriers, were pooled for the age effect calculations and the discriminant analyses.

VWF:Ag levels. The values for VWF:Ag as assessed by the Laurell method are shown in Table 4. The mean for the 137 normal women (first column) was 101 U per deciliter. As with F.VIII:C, there was a significant effect of age and institution on the level of VWF:Ag. When the means were adjusted for age and institution, there was not a significant difference between the types of carriers ($P = .83$) or between pooled carriers and normals ($P = .16$). The VWF:Ag values of all carriers were pooled for subsequent calculations.

Effect of age on F.VIII:C and VWF:Ag levels. The effect of age on F.VIII:C and VWF:Ag levels was examined in both normal women and carriers. Logarithmically transformed values were regressed on a cubic function of age. Although both linear and quadratic, but not cubic, terms were required to obtain a satisfactory fit over the range 10 to 60 years of age, the range from 18 to 50 years of age could be fitted by a linear function. The best fitting quadratic functions of age for the normals and the carriers were the following: F.VIII:C for normal subjects was $\mathrm{Ln(F.VIII{:}C)} = .000360(\mathrm{age})^2 - .0209(\mathrm{age}) + 4.78$; for carriers, it was $\mathrm{Ln(F.VIII{:}C)} = .000317(\mathrm{age})^2 - .0193(\mathrm{age}) + 4.15$. VWF:Ag for normal subjects was $\mathrm{Ln(VWF{:}Ag)} = .000422(\mathrm{age})^2 - .0225(\mathrm{age}) + 4.65$; for carriers, it was $\mathrm{Ln(VWF{:}Ag)} = .000277(\mathrm{age})^2 - .0170(\mathrm{age}) + 4.76$.

Table 2. Subjects in Study

Laboratory	Normal Women	Paternal Carriers	Maternal Carriers	Sporadic Carriers	Total
Oxford	21	27	20	8	76
Chapel Hill	—	34	47	9	90
Los Angeles	20	20	20	—	60
Milan	49	20	19	10	98
Chicago	—	5	8	—	13
Malmo	30	16	15	10	71
Leiden	17	20	21	—	58
Ibadan	—	—	4	3	7
Total	137	142	154	30	473

Table 3. Factor VIII:C Levels

Laboratory	Normal Women	Paternal Carriers	Maternal Carriers	Pooled Carriers
Oxford	92 ± 16	51 ± 14	53 ± 15	52 ± 14
Chapel Hill	—	54 ± 22	50 ± 33	52 ± 29
Los Angeles	110 ± 44	53 ± 23	62 ± 25	57 ± 29
Milan	117 ± 49	54 ± 25	51 ± 21	52 ± 23
Chicago	—	62 ± 20	71 ± 30	68 ± 28
Malmo	109 ± 43	60 ± 30	61 ± 25	64 ± 28
Leiden	84 ± 24	48 ± 21	47 ± 26	48 ± 24
Ibadan	—	—	72 ± 29	72 ± 29
Average of means	106 ± 42	54 ± 26	54 ± 24	55 ± 27

Levels are standard plasma = 53 IU per deciliter. Values in tables are shown in "working" units, ie, values obtained × 0.64. Values are mean ± SD.

Although the means for F.VIII:C in normal subjects and carriers were significantly different at all ages as expected ($P < .0001$), the age regression coefficients were not significantly different ($P > .3$). The mean VWF:Ag values were not different between normal subjects and carriers, although both showed an effect of age. Neither were the age regression coefficients on the transformed data different for normal subjects or carriers ($P > .05$).

Effect of ABO blood group on F.VIII:C and VWF:Ag levels. ABO blood types were available on most of the subjects of four laboratories. The frequencies of the blood types were: (among 54 normal subjects) O = 0.50, A = 0.362, B = 0.12, and AB = 0.017; and (among 198 carriers) O = 0.49, A = 0.38, B = 0.10, and AB = 0.03. These frequencies are typical of West European peoples.[29] Approximately 75% of non-Os are type A, and types B and AB appear to have somewhat larger effects on the variables concerned than does type A[25,26]; therefore, the subjects are grouped into two classes: O and non-O. The data relating F.VIII:C and VWF:Ag to ABO blood type are shown in Table 5 as comparisons between O and non-O subjects stratified for carriership and examined separately for F.VIII:C and VWF:Ag. In 13 of the 14 possible comparisons, the levels are higher in the non-O member of a pair. Using the data in Table 5, the differences in weighted averages (non-O > O) were for F.VIII:C, 22.9 U per deciliter in normal subjects and 10 U per deciliter in carriers. Comparable results for VWF:Ag were: 25.8 U/dL in normal subjects and 34.6 U/dL in carriers.

The significance of the difference between O and non-O subjects for F.VIII:C and VWF:Ag was confirmed using a general linear model which included age and laboratory as covariates ($P < 0.01$).

Table 4. VWF:AG Levels

Laboratory	Normal Women	Paternal Carriers	Maternal Carriers	Pooled Carriers
Oxford	86 ± 31	95 ± 43	116 ± 34	104 ± 41
Chapel Hill	—	110 ± 57	107 ± 87	108 ± 76
Los Angeles	122 ± 44	124 ± 69	125 ± 34	124 ± 53
Milan	107 ± 61	99 ± 36	103 ± 38	101 ± 36
Chicago	—	123 ± 54	116 ± 77	119 ± 67
Malmo	85 ± 35	84 ± 24	105 ± 50	94 ± 40
Leiden	103 ± 27	110 ± 38	110 ± 41	110 ± 40
Ibadan	—	—	151 ± 64	151 ± 64
Average of means	101 ± 47	105 ± 49	112 ± 60	108 ± 47

Levels of standard plasma activity arbitrarily set at 100 U/dL. Values are means ± SD.

DISCUSSION

Despite the fact that each of the participating laboratories collected blood somewhat differently and used similar though not identical assays, it was believed that the geographically separate groups of carriers could be collated for analysis, since all were based on the same standard. This lyophilized plasma has essentially the properties of plasma #80/511, now adopted by the WHO as the First International Reference Preparation for Factor VIII Related Activities in Plasma,[30] and is very stable at temperatures below −30 °C.

A major variable in the study was the difference in mean levels of F.VIII:C and VWF:Ag between laboratories. Klein and colleagues[31] ascribed similar interlaboratory variation largely to differences in laboratory technique, because the same group of women was examined in a single laboratory setting by different technicians using a single standard. Barrowcliffe and colleagues[30] and we have observed significant differences in the means between laboratories when a single standard was used on separate small populations.

The data of Klein and co-workers[31] clearly document that differences in technique between laboratory workers is an important source of variation in carrier detection. Their study involved a single population and they were, therefore, unable to assess variation between populations. The present study was of separate small populations, and the interlaboratory variation which persisted after correction for age and ABO blood type could reflect population differences as well as differences in technique. There are several obvious genetic reasons why population differences might be encountered: (a) the normal F.VIII locus of each carrier will be occupied by any one of several isoalleles with different effects,[11] and the frequencies of the isoalleles may vary between small populations; (b) the frequencies of the isoalleles of (unspecified) modifying genes at other loci may vary; and (c) the average ratio of lyonization between the normal and abnormal alleles of groups of heterozygotes may not be the same.

The original goals of this study were narrow, ie, to determine whether there is a difference in F.VIII:C levels between "paternal" and "maternal" carriers and whether age has an effect on levels of F.VIII:C and VWF:Ag. Answers were obtained on both. The results show quite clearly that there is no difference between the levels of F.VIII:C and VWF:Ag of "paternal" and "maternal" carriers. The populations that had produced opposite results had been much smaller than that of this study, and it is likely that some of the groups had not been representative of carriers generally. Second, age does affect levels of F.VIII:C and VWF:Ag, although not as much as blood type. Because significant age effects were also found in the twin study of Orstavik and co-workers,[26] age must be regarded as a variable to be taken into account in hemophilia carrier detection.

This study confirms that women of blood type O have lower F.VIII:C and VWF:AG levels than do women who are

Table 5. ABO Blood Group and Factors VIII:C and VWF:Ag in Normal Women and Carriers of Hemophilia A

		Normal Subjects						Carriers					
		Factor VIII:C			VWF:Ag			Factor VIII:C			VWF:Ag		
Laboratory	Statistic	O		Non-O	O		Non-O	O		Non-O	O		Non-O
Chapel Hill	Mean							50.6		64.7	79.9		132.2
	SD	—		—	—		—	30.7	v	36.0	48.3	v	87.9
	(No.)							(42)		(46)	(42)		(46)
Oxford	Mean	76.0		102.2	61.7		91.5	54.5		60.8	86.2		120.7
	SD	5.7	v	12.3	15.8	v	30.3	18.6	v	17.1	29.8	v	40.3
	(No.)	(6)		(13)	(6)		(13)	(27)		(29)	(27)		(29)
Leiden	Mean	75.7		97.8	88.7		116.0	42.6		53.2	98.1		118.5
	SD	10.7	v	30.6	15.1	v	29.4	15.5	v	28.4	33.9	v	42.2
	(No.)	(8)		(9)	(8)		(9)	(16)		(22)	(16)		(22)
Los Angeles	Mean	97.3		132.7	97.6		144.7	49.6		51.5	143.0		121.7
	SD	31.3	v	27.4	38.5	v	30.5	17.8	v	16.2	85.0	v	27.8
	(No.)	(11)		(7)	(11)		(7)	(9)		(7)	(9)		(7)

not of type O, and that this is a significant variable that affects carrier detection. The physiological relationship between ABO type and the level of these clotting factors is obscure. Highly purified preparations of F.VIII containing ABO blood group substance have been described,[32] but this does not necessarily mean that it is a functionally important part of the F.VIII complex. The genetic information tends to suggest the opposite in fact, ie: (a) the ABO types are present when F.VIII:C and VWF:Ag are absent[33]; (b) the genes for ABO and VWD are not linked in the usual sense[34]; and (c) the gene for F.VIII:C is on the X-chromosome whereas the ABO locus is on chromosome 9. Nevertheless, F.VIII:C and VWF:Ag levels are clearly lower in persons of blood type O, a variable included in the discriminant function published by Winter and co-workers.[14] Orstavik and colleagues[26] concluded that the ABO blood group has a greater genetic effect on levels of F.VIII:Ag and VWF:Ag in normal persons than does any other genetic mechanism, including isoalleles at the F.VIII:C locus. Their data suggested that the primary effect of ABO is on the level of VWF:Ag and that the effect on F.VIII:C is secondary. How these independent genes interact is unknown, but it might occur posttranslationally through interaction of their products. The type of ABO substance on the cell membrane for instance, may influence the rate at which the clotting factors exit from the cell. But there are many other possibilities.

Two other matters that were of concern to the WHO Committee in 1977[8] were not studied, but it seems worthwhile to note them. They wished to determine whether carriership can be detected accurately in women who are already pregnant when first seen. Subsequent studies by Mibashan and colleagues,[35] Hoyer and colleagues[36] and Barrow and colleagues[10] have demonstrated that the procedures recommended by the WHO are satisfactory for pregnant carriers who can be detected without difficulty at least until the 22nd week of gestation.

They also wished to discover whether the taking of oral contraceptives confounds carrier detection. Stableforth and colleagues[37] and McCallum and colleagues[25] have reported that the use of oral contraceptives does not affect levels of F.VIII:C and VWF:Ag. This was not a matter of importance in the present study, since only 5 of 473 subjects (1.1%) reported using this form of birth control. This may well be an underestimate of the true frequency of contraceptive use by all women, however, since many of our subjects were unmarried, prepubertal, or postmenopausal.

Kobrinsky and co-workers[38] have reported that in plasma samples obtained one hour after the administration of a small dose of DDAVP there is a comparable rise of VWF:Ag in both carriers and normal women, whereas the increase in F.VIII:C is less among carriers. The rate of misclassification on single testing after DDAVP administration in their small sample was ~5%, comparable to the rate found earlier with triple testing using the ordinary procedure.[5] If this is confirmed, administration of DDAVP together with corrections for age, ABO blood type, and laboration variation might greatly enhance the likelihood of correctly classifying hemophilia carriers by phenotypic methods.

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REFERENCES

1. Merskey C, Macfarlane RG: The female carrier of hemophilia. A clinical and laboratory study. Lancet 1:487, 1951

2. Graham JB, McLendon WW, Brinkhous KM: Mild hemophilia: An allelic form of the disease. Am J Med Sci 225:46, 1953

3. Veltkamp JJ, Drion EF, Loeliger EA: Detection of the carrier state in hereditary coagulation disorders. Thromb Diath Haemorrh 19:403, 1968

4. Zimmerman TS, Ratnoff OD, Littell AS: Detection of carriers of classic hemophilia using an immunologic assay for antihemophilic factor (factor VIII). J Clin Invest 50:255, 1971

5. Elston RC, Graham JB, Miller CH, Reisner HM, Bouma BN: Probabilistic classification of hemophilia A carriers by discriminant analysis. Thromb Res 8:683, 1976

6. Reisner HM, Katz HJ, Goldin LR, Barrow ES, Graham JB: Use of a simple visual assay of Willebrand factor for diagnosis and carrier detection. Br J Haematol 40:339, 1978

7. Wahlberg T, Blomback M, Brodin U: Carriers and noncarriers of haemophilia A: I. Multivariate analysis of pedigree data, blood coagulation tests and factor VIII variables. Thromb Res 25:401, 1982

8. Akhmeteli MA, Aledort L, Alexaniants S, Bulanov AG, Elston RC, Ginter EK, Goussev A, Graham JB, Hermans J, Larrieu MJ, Lothe F, McLaren AD, Mannucci PM, Prentice CRM, Veltkamp JJ: Methods for detection of hemophilia carriers: A memorandum. Bull WHO 55:675, 1977

9. Barrow ES, Miller CH, Reisner HM, Graham JB: Genetic counseling in haemophilia by discriminant analysis, 1975–1980. J Med Genet 19:26, 1982

10. Graham JB, Elston RC, Barrow ES, Reisner HM, Namboodiri KK: Statistical methods for carrier detection in the hemophilias, in Bloom AL (ed): Methods in Hematology: The Hemophilias. Edinburgh, Churchill-Livingston, 1982

11. Filippi G, Mannucci PM, Coppola AF, Rinaldi A, Siniscalco M: Studies on hemophilia A in Sardinia bearing on the problems of multiple allelism, carrier detection and differential mutation rate in the two sexes. Am J Hum Genet 36:44, 1984

12. Graham JB, Green PP, McGraw RA, Davis LM: Application of molecular genetics to prenatal diagnosis and carrier detection in the hemophilias: Some limitations. Blood 66:759, 1985

13. Jeremic M, Weisert O, Gedde-Dahl TW: Factor VIII (AHG) levels in 1016 regular blood donors. Scand J Clin Lab Invest 36:461, 1976

14. Winter RM, Tuddenham EGD, Goldman E, Matthews KB: A maximum likelihood estimate of the sex ratio of mutation rates in hemophilia A. Hum Genet 64:156, 1983

15. Preston AE, Barr A: The plasma concentration of factor VIII in the normal population. II. The effects of age, sex and blood group. Br J Haematol 10:238, 1964

16. Seligsohn U, Zivelin A, Perez C, Modan M: Detection of

hemophilia A carriers by replicate factor VIII activity and factor VIII antigenecity determinations: Br J Haematol 42:433, 1979

17. Wahlberg TB, Savidge GF, Blomback M, Wiechel B: Influence of age, sex, and blood groups on 15 blood coagulation laboratory variables in a reference material composed of 80 blood donors. Vox Sang 39:301, 1980

18. Biggs R, Rizza CR: The sporadic case of haemophilia A. Lancet 2:431, 1976

19. Chediak J, Telfer MC, Joajaroenkul T, Green D: Lower factor VIII coagulant activity in daughters of subjects with haemophilia A compared to other obligate carriers. Blood 55:552, 1980

20. Graham JB: Blood 56:742, 1980 (letter)

21. Kasper CK: Blood 56:742, 1980 (letter)

22. Lusher JM, Warrier AI, Khalifer AS: Blood 56:1153, 1980 (letter)

23. Jones PK, Ratnoff OD: Sources of variability in antihemophilic factor (factor VIII) procoagulant titers and precipitating antigen levels among obligate carriers of classic hemophilia. Blood 57:928, 1981

24. Colonia VJ, Roisenberg I: Investigation of associations between ABO blood groups and coagulation, fibrinolysis, total lipids, cholesterol and triglycerides. Hum Genet 48:221, 1979

25. McCallum CJ, Peake IR, Newcombe RG, Bloom AL: Factor VIII levels and blood group antigens. Thromb Haemost 50:757, 1983

26. Orstavik KH, Magnus P, Reisner HM, Berg K, Graham JB, Nance W: Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. Am J Human Genet 37:89, 1985

27. Green PP, Mannucci PM, Briet E, Ljung R, Kasper CK, Essien EM, Rizza CR, Graham JB. Carrier detection in hemophilia "A," a co-operative international study, II: The efficiency of linear discriminants. Blood (this issue)

28. Laurell C: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 15:45, 1966

29. Thompson JS, Thompson MW: Genetics in Medicine (ed 3). Philadelphia, Saunders, 1980

30. Barrowcliffe TW, Tydeman MS, Kirkwood TBL, Thomas DP: Standardization of factor VIII–III. Establishment of a stable reference plasma for factor VIII-related activities. Thromb Haemost 50:690, 1983

31. Klein HG, Aledort LM, Bouma BN, Hoyer LW, Zimmerman TS, DeMets DL: A co-operative study for the detection of the carrier state of classic hemophilia. N Engl J Med 296:959, 1977

32. Sodetz JM, Paulson JC, McKee PA: Carbohydrate composition and identification of blood group A, B, and H oligosaccharide structures on human factor VIII/von Willebrand factor. J Biol Chem 254:10754, 1979

33. Miller CH, Graham JB, Goldin LR, Elston RC: Genetics of classic von Willebrand's disease. I: Phenotypic variation within families. Blood 54:117, 1979

34. Verp MS, Rudvany RM, Conneally PM, Patel VA, Martin AO, Simpson JL: Linkage analysis in von Willebrand disease. Clin Genet 24:434, 1983

35. Mibashan RS, Peake IR, Newcombe RG, Thumpston JK, Gorer R, Furlong RA, Rodeck CH: Carrier detection of haemophilia A in pregnancy by measurement of VIII:C/RAg and VIII:CAg/RAg ratios. Thromb Haemost 46:187, 1981

36. Hoyer LW, Çarta CA, Mahoney MJ: Detection of hemophilia carriers during pregnancy. Blood 60:1407, 1982

37. Stableforth P, Montgomery DC, Wilson E, Churchill WGL, Dormandy K, Hardisty RM: Effect of oral contraceptives on factor VIII clotting activity and factor VIII related antigen in normal women. J Clin Pathol 28:498, 1975

38. Kobrinsky NL, Watson CM, Cheang MS, Bishop AJ: Improved hemophilia A carrier detection by DDAVP stimulation of factor VIII. J Pediatr 104:718, 1984

Carrier Detection in Hemophilia A: A Cooperative International Study. II. The Efficacy of a Universal Discriminant

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Factor VIII (F.VIII) and von Willebrand factor (VWF):Ag data collected by eight laboratories on a total of 336 obligatory carriers of hemophilia A and 137 normal women were used to answer several questions concerning the construction of linear discriminants for carrier detection. It was found: (a) that a "universal" linear discriminant can be constructed which is suitable for use in all laboratories and is nearly as effective as laboratory-specific discriminants; (b) that inclusion of age and ABO blood type data improved the efficacy of these discriminants; (c) that substitution of alternative assays for F.VIII and VWF:Ag did not generally improve the efficacy of the discriminants over that obtained using the bioassay for F.VIII:C and Laurell's immunoassay for VWF:Ag; (d) that linear discriminants were far more effective than discriminants based on the F.VIII:C/VWF:Ag ratio. A step-wise procedure is given which any laboratory may follow in using the universal discriminant for carrier detection.

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LINEAR discriminants which incorporate data on (Factor VIII) F.VIII:C and (von Willebrand factor) VWF:Ag levels* are well established tools for estimating the probability of carriership of hemophilia A,[1-3] and together with newer but less widely applicable recombinant DNA techniques they are likely to remain important in genetic counseling for many years.[4,5] The exact manner in which the assay data are combined to give the discriminant value depends on a mathematical expression whose exact form may vary, but which is to be chosen so that the populations of carrier and of normal women have as little overlap of their discriminant values as possible. Elston and colleagues[2] suggested that interlaboratory differences in assay methods and/or reference populations make it necessary for each laboratory to construct its own discriminant. However, some laboratories may not have access to a large enough carrier population to permit the calculation of an accurate discriminant.

In the present study, data collected in a multicenter international study involving eight laboratories have been used to examine in detail the feasibility of a common discriminant for use in multiple laboratories. The common thread linking the studies of the different laboratories was a single lyophilized plasma standard.[6] The form that such a "universal" discriminant should take to optimize discrimination was also examined, since no clear consensus has emerged from previous studies. The specific issues considered here include: the importance of correcting for age and for ABO blood group, both of which have appreciable effects on F.VIII:C and VWF:Ag levels in both normal subjects and carriers[2,3,6-8]; the effect of correcting for differences in mean values between laboratories; whether assays other than the customary bioassay for F.VIII:C and Laurell's electroimmunoassay for VWF:Ag can significantly improve discrimination; and the relative accuracy of the widely used ratio method for discrimination.[9-11]

MATERIALS AND METHODS

The motivation and the design of the study are described more fully in our companion article.[6] In brief, eight different laboratories assayed a total of 336 obligatory carriers and 137 normal women for F.VIII:C and VWF:Ag levels, each laboratory using a lyophilized plasma standard provided by the Oxford Haemophilia Centre, but otherwise using its own "in-house" methods. No subjects were pregnant, and only five were using oral contraceptives. Because no differences were detected between "maternal" and "paternal" carriers,[6] their data were pooled with those of "sporadic" obligatory carriers for the purposes of computing the discriminants and determining misclassification rates and average odds.

Statistical methods for computing covariate-adjusted discriminants were for the most part the same as previously described.[2] F.VIII:C and VWF:Ag values were logarithmically transformed to reduce their skewness and that of the discriminants computed from them. Initial regression studies[6] indicated that a quadratic term was necessary to correct fully for age effects on both F.VIII:C and VWF:Ag, and that although the mean levels of the laboratories differed, the laboratories did not differ in the effect which age had on their carrier populations. Furthermore, ABO blood type was found to have a significant effect on F.VIII:C and especially on VWF:Ag.[6] Linear age-adjusted discriminants were computed for each laboratory and for the entire (pooled) sample, according to the method described by Elston and co-workers.[2] To assess the effect on the discriminant of individuals with extreme ages, discriminants were also computed using only individuals with ages between 18 and 50. For the laboratories having data on ABO blood type (four of eight), discriminants were constructed which adjust for this variable in addition to age; the procedure for doing this is identical with that

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*Following recent nomenclature conventions, F.VIII:C will be used to designate the clotting activity which is missing in severe hemophilia A measured by bioassay, and F.VIII:Ag (previously FVIII C:Ag) to designate it when determined immunologically. VWF:Ag (previously VIII R:Ag) refers to the antigen that is absent in severe von Willebrand's disease.

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described for age-adjusted discriminants by Elston and co-workers,[2] except that in each regression a class variable with two classes (types O and Non-O) is included in addition to the linear and quadratic age terms. For the three laboratories performing alternative assays for F.VIII:C and VWF:Ag in addition to the customary ones, discriminants were constructed using these alternative data. Finally, discriminants were computed based on the F.VIII:C/VWF:Ag ratio.[9-11] Quadratic discriminants, which have occasionally been used in other laboratories,[12] were not computed on this data set, because they are quite sensitive to how the data are transformed and have other undesirable properties.[13]

For each type of discriminant, two measures of efficacy were computed, the "average odds" and the "misclassification rate." The odds for carriership were computed for each carrier based on the value of her discriminant. Odds greater than 100:1 were "trimmed" to 100:1, to make the summary measures more robust to outliers, although some calculations were also done using untrimmed odds to determine how individuals with very high odds might affect the results. The (geometric) average of the odds for carriership was then computed for all carriers in the sample. High average odds indicate that, on average, the discriminant assigns a high probability of carriership to carriers. As is pointed out in the WHO Memorandum on carrier detection,[14] a *t* test for comparing the efficacy of two discriminants may be performed using the logarithm of the ratio of their average odds. The other method of evaluation, "misclassification rate" for carriers, is simply the percentage of "misclassified" carriers in the sample, in which a carrier is arbitrarily considered misclassified if her odds for carriership are $<1:1$. A similar procedure was used to compute average odds and misclassification rates for the normal controls, misclassified normal controls being those with odds for carriership $>1:1$.

From the viewpoint of genetic counseling, average odds are clearly a more sensitive and useful measure of the behavior of a discriminant than is the cruder misclassification rate since they take into account the magnitude of the probabilities assigned to individuals, rather than the relations of individual values to an arbitrary "cut-off" point. The misclassification rate is useful, however, in assessing the behavior of discriminants for individuals "on the borderline," ie, having odds of $\sim 1:1$. Accordingly, we have included both summary measures in Tables 1 through 5.

Ideally, a sample used to estimate a discriminant should not contain the subjects who are going to be used to judge its efficacy, since, if it does, the resulting estimate of the misclassification rate (or average odds) will be biased. Consequently, the following method was used to obtain an unbiased estimate of the accuracy of a pooled, or "universal," discriminant. For each laboratory, a discriminant was computed using data pooled from the other seven laboratories. This discriminant was then used to compute odds for carriership of, and to classify, each individual from the given laboratory's sample, and the average odds and misclassification rates for that laboratory. Total misclassification rates and average odds were produced by taking a weighted average over all laboratories. This method is analogous to the "leaving one out" (or "jack-knife") method proposed by Lachenbruch,[15,16] but is computationally far easier to implement in the case of a covariate-adjusted discriminant. Furthermore, it more directly addresses the issue of interest here; namely, whether a discriminant computed from data produced in one group of laboratories can validly be applied to that of other laboratories. The average odds and misclassification rates reported below for the "universal" discriminant were obtained by this method and are, therefore, unbiased. The values reported for the "laboratory-specific" discriminants, however, were computed using the sample from which the discriminant was generated and are therefore somewhat biased.

Sample sizes varied, because the data base is not homogeneous, lab by lab. All eight laboratories provided data on carriers whereas only five provided a normal cohort. Four laboratories provided ABO blood group data, but only three of these also provided a normal cohort. For each discriminant, the average odds are greater for carriers than for normal subjects, and the rate of misclassification is greater for carriers than for normal subjects; both tendencies are due to the fact, itself a consequence of "lyonization,"[17] that the distribution of carriers' values for the discriminant has a higher variance than the distribution of normals' values.

Computer programs to compute covariate adjusted discriminants, average odds, and misclassification rates, were written in SAS language and run on an IBM 3081 computer.

RESULTS

To investigate the general feasibility of a "universal" discriminant, we examined what appeared to be the pertinent variables from analyses described in our companion article.[6] These were age, ABO blood group, and interlaboratory variation in mean levels. First, the efficacies of laboratory-specific discriminants computed with and without corrections for age and ABO blood group were compared. These were initially done using "untrimmed" odds and then repeated using the "trimmed" odds. The results with the trimmed odds are shown in Table 1.

The analyses using "untrimmed" odds (not shown) indicated that correction for age and ABO blood group significantly improved the average odds. This improvement seems to have come primarily from individuals having relatively large odds, since when "trimmed" odds are used the improvement vanishes entirely for age correction alone and is relatively small for the combined age and ABO blood group correction, and since the corrections do not improve misclassification rates (Table 1, lines 1 through 3). The (unbiased) estimates obtained with the universal discriminants using "trimmed" odds (cf. lines 4 and 5) show a similar pattern.

To determine whether individuals with extreme ages were having large effects on the discriminants, we recomputed the laboratory-specific discriminants using only individuals between the ages of 18 and 50 years (lines 6 through 8). The samples were smaller, and the results were inconsistent; with respect to the age and ABO adjusted discriminant, the average odds and the misclassification rates improved for normals when the truncated age range was used, but they worsened for carriers (line 8 *v* line 3).

We then investigated (Table 2) the effect of interlaboratory variation, since there are significant differences in mean levels of F.VIII:C and VWF:Ag between laboratories.[6] Not surprisingly, the laboratory-specific discriminant is appreciably more efficient than the standard "universal" discriminant when adjusted for age and ABO only (cf lines 1 and 2). This could be due in part to the intrinsic bias in average odds and misclassification rates of laboratory-specific discriminants, but it probably also reflects local differences in assay methods and/or carrier populations.

To see whether correction for interlaboratory variation in mean levels would improve the efficacy of the "universal" discriminant, two adjustments were tried. First, the mean levels of the log-transformed F.VIII:C and VWF:Ag values for the carriers in each laboratory were computed and were subtracted from the level of each person (carrier or normal)

Table 1. Effect of Adjustment for Age and ABO Blood Group

Discriminant (Adjustment)	Carriers			Normal Subjects			Total	
	N	Average Odds Favoring Carriership	Misclassification Rate (%)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	Misclassification	%
Lab-specific (unadjusted)*	226	27:1	11.5	136	1:7.9	2.9	30/362	8.2
Lab-specific (age)*	226	22:1	14.2	136	1:8.8	2.9	36/362	9.9
Lab-specific (age, ABO)†	109	38:1	11.9	54	1:25	3.7	15/163	9.2
Universal (unadjusted)‡	196	11:1	19.9	54	1:3.2	9.3	44/250	17.6
Universal (age, ABO)‡	196	13:1	17.3	54	1:3.6	7.4	38/250	15.2
Lab-specific§ (unadjusted)*	126	26:1	10.3	100	1:9	4.0	17/226	7.5
Lab-specific§ (Age)†	126	26:1	12.7	100	1:10	3	19/226	8.4
Lab-specific§ (Age, ABO)†	60	27:1	15	42	1:34	0	9/102	8.8

*Subsample consists of five laboratories that supplied a normal cohort.

†Subsample consists of 3 laboratories supplying ABO data and a normal cohort.

‡Subsample on which ABO data were available.

§Computed using subsample consisting of women between 18 and 50 years of age.

in that laboratory's sample. This had the effect of adjusting the carrier population of each laboratory to a mean of 0, while preserving the relative differences between carriers and normals. Age and ABO-adjusted universal discriminants were then computed in the usual manner with these data. The resulting average odds and misclassification rates are given in line 3 of Table 2. The performance of the "universal" discriminant was improved when adjusted for differences between laboratories in this manner (line 1 *v* line 3), although it does not reach the efficacy of the lab-specific discriminants (line 2).

The adjustment procedure was then repeated using the

Table 2. Effect of Adjustment for Laboratory Variation

Discriminant (Adjustment)	Carriers			Normal Subjects			Total	
	N	Average Odds Favoring Carriership	Misclassification Rate (%)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	Misclassification	%
Universal (Age, ABO)*	196	13:1	17.3	54	1:3.6	7.4	38/250	15.2
Lab-specific (age, ABO)†	109	38:1	11.9	54	1:25	3.7	15/163	9.2
Universal (age, ABO, carrier mean)*	196	19:1	14.3	54	1:4.1	9.3	33/250	13.2
Universal (age, ABO, normal mean)†	109	31:1	10.1	54	1:9.8	5.6	14/163	8.5
Universal (age)‡	331	11:1	16.9	136	1:4.5	8.1	67/467	14.3
Lab-specific (age)§	226	22:1	14.2	136	1:8.8	2.9	36/362	9.9
Universal (Age, carrier mean)‡	331	15:1	14.5	136	1:4.5	6.6	57/467	12.2
Universal (Age, normal mean)§	226	19:1	13.3	136	1:6.2	2.9	34/362	9.4

*Subsample on which ABO data were available.

†Subsample consists of three laboratories supplying ABO data and a normal cohort.

‡Total sample.

§Subsample consists of five laboratories providing a normal cohort.

mean of the normal subjects from each laboratory's sample to adjust that laboratory's data. The result is seen in line 4. The improvement in the performance of the "universal" discriminant is clear; the misclassification rate is slightly *lower* than that of the laboratory-specific discriminant (line 2). This is especially remarkable when one considers that the estimate of misclassification rate for the lab-specific variety is known to be biased downward. The average odds for the adjusted universal discriminant are comparable to those of the laboratory-specific discriminants for carriers (line 4 *v* line 2), although remaining somewhat worse for normal subjects. A clearer picture of the relative performance of these two discriminants may be obtained from the scatterplot of the odds they assign to carriers (Fig 1A). Points above the diagonal correspond to individuals to whom the universal discriminant assigns higher odds for carriership (and thus does a better job of classifying), whereas those below the diagonal are assigned higher odds by the lab-specific discriminant. Points in the upper right hand quadrant correspond to individuals classified correctly by both discriminants. Figure 1B is the corresponding plot for normals, but the interpretation must be reversed since the odds plotted are odds for carriership: points above the diagonal are now better classified by the lab-specific discriminant, and the points

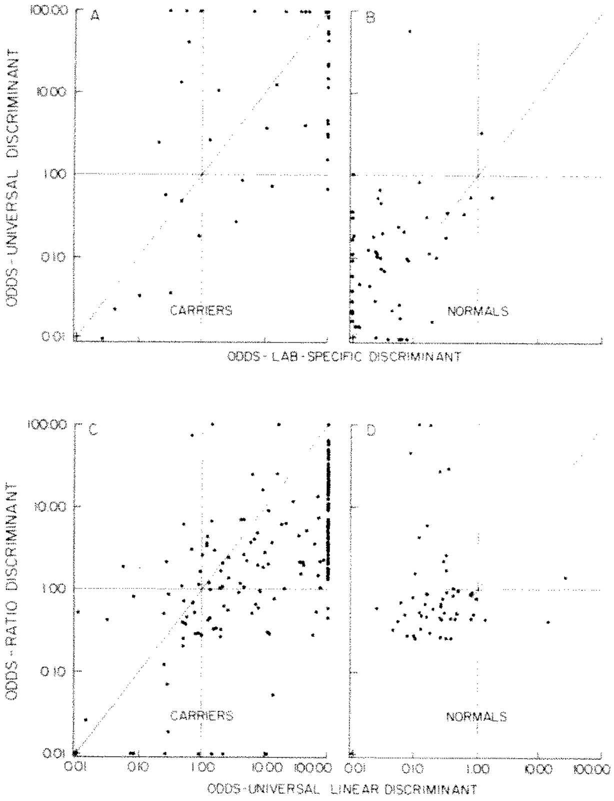

Fig 1. Comparisons of the universal linear discriminant with the lab-specific linear discriminant (A, B), and of the universal ratio discriminant with the universal linear discriminant (C, D). All discriminants were adjusted for age and ABO blood type; the universal linear discriminant in A and B is normal-mean-adjusted, but that in C and D is not. Except as noted, each solid circle represents an individual. The coordinates are scaled in "odds favoring carriership," where 0.01 = 1:100 and 100.00 = 100:1. The diagonal indicates equal odds for both discriminants at any point, so that the location of a point with respect to the diagonal indicates which method was more efficient. The vertical and horizontal lines producing the four quadrants provide the opportunity to assess efficacy quickly, the upper right and lower left being most indicative. (A) Comparison of the universal and lab-specific linear discriminants for carriers. Note that the lab-specific discriminant classifies carriers overall slightly more efficiently than the universal. The points in the right upper quadrant are those classified correctly by both discriminants; the extreme upper right-hand point represents 64 carriers assigned odds >100:1 by both discriminants. The points in the left lower quadrant are those misclassified by both discriminants. (B) Comparison of universal and lab-specific discriminants for normal subjects. The high concentration of points in the lower right quadrant means that both discriminants are effective, lab-specific tests producing lower odds in most instances. (C) Comparison of the (universal) ratio discriminant with the (universal) linear discriminant for carriers. A moderate number of persons were incorrectly classified by both tests (left lower quadrant). The majority were correctly classified by both tests (right upper quadrant), the linear discriminant being generally more effective. More were correctly classified by the linear and incorrectly classified by the ratio (lower right quadrant) than were correctly classified by the ratio and incorrectly classified by the linear discriminant (upper left quadrant). The concentration of points along the margin of the right upper quadrant represent carriers whose odds were ≥100:1 with the linear discriminant and <100:1 with the ratio. (A similar but lesser tendency is seen in the right upper quadrant of A and the left lower quadrant of B). (D) Comparison of ratio discriminant and linear discriminant for normal subjects. The linear discriminant is clearly more effective overall. Points in the left lower quadrant are those correctly classified by both discriminants. Those in the left upper quadrant are persons correctly classified by the linear discriminant but incorrectly by the ratio. Those in the right lower quadrant were correctly classified by the ratio but incorrectly by the linear discriminant.

classified correctly by both discriminants are those in the lower left quadrant. The scatterplots reinforce the conclusions obtained from the average odds: the discriminants perform equally well on carriers, but on normal subjects the lab-specific discriminant has a slight edge.

That these results were not a peculiarity of the laboratories supplying the ABO data was verified by repeating the analyses on the entire sample, using age-adjusted- but not ABO-adjusted discriminants. The results (lines 5 through 8 of Table 2) were quite similar to those of lines 1 through 4. Here again, the best discrimination using the universal discriminant was achieved after adjusting the test values using the means of a normal cohort (line 8).

The effect of using alternative assays to measure F.VIII and VWF:Ag levels was then investigated. In Table 3, all VWF:Ag assays were done by Laurell's electroimmunoassay method, whereas F.VIII was measured either by bioassay (F.VIII:C) or immunoassay (F.VIII:Ag). The pattern is not consistent. In Milan, for instance, better discrimination is obtained by using F.VIII:C than F.VIII:Ag, but in Malmo the reverse is true. In Leiden, the discriminant with F.VIII:Ag has better average odds, but the same misclassification rate, as that with F.VIII:C.

The utility of alternative assays for VWF:Ag is examined in Table 4, Milan providing the relevant data. Measurement of VWF:Ag by immunoradiometric assay (IRMA) or enzyme-linked immunosorbent assay (ELISA) did not basically improve the accuracy of the discriminant based on data obtained by EIA.

Finally, in Table 5, the relative accuracy of linear discriminants is compared with that of discriminants based on the F.VIII:C/VWF:Ag ratio. Linear discriminants are clearly superior to ratio discriminants in all comparisons, most strikingly in the proportion of false-positives (normal subjects misclassified as carriers). All the "universal" ratio discriminants performed quite poorly, the average odds in line 4 actually being <1:1. The latter anomaly is due in part to the presence of five carriers having a F.VIII:C/VWF:Ag ratio > 4 to whom the ratio discriminant consequently assigns extremely low odds for carriership. When these individuals are eliminated from the sample, the average odds for carriership improves to 2.9:1, still significantly worse than that for the linear discriminant. The superiority of the linear discriminant is also evident from scatterplots of the odds (Fig 1C and 1D).

DISCUSSION

We have found an effective "universal" discriminant that can be used in any laboratory. Although laboratory-specific discriminants are possibly more effective for individual laboratories, their greater effectiveness was found to be due almost entirely to the fact that laboratories differ significantly in their mean levels of F.VIII:C and VWF:Ag. The latter may result from genetic differences in local populations or systematic differences in laboratory technique. Whatever the cause, when these mean differences among laboratories are removed, the resulting "universal" discriminant is just as effective as are the laboratory-specific ones when judged by the criterion of misclassification rates and very nearly as effective when judged by the criterion of average odds.

We suggest, therefore, that the step-by-step procedure given in Appendix A, including the specific constants we have derived, can be used effectively for carrier detection in any laboratory if a local set of normal subjects is assayed for F.VIII:C and VWF:Ag against the laboratory standard, and their mean values are computed and used for adjustment as we have described. It is not necessary that each laboratory base its assays on the International Standard, unless there is the additional goal of comparing potencies between laboratories or combining data for subsequent calculations.

As detailed in our companion article,[6] both age and ABO blood type have an effect on F.VIII:C and VWF:Ag levels; blood type has a greater effect than age. Although correction for these variables does not greatly affect the average efficacy of the discriminant, we recommend that it be done since it may have a substantial effect for some individuals and is, in any case, computationally trivial (Appendix A). Use of a truncated age range did not improve the efficacy of the discriminant.

There have been reports[9,18] that determining F.VIII:C by immunoassay (F.VIII:Ag) gives better discrimination than does use of bioassays. Our data from three laboratories show that there is not a real difference between the utility of the

Table 3. Effect of Different Methods for Measuring Factor VIII

	Carriers			Normal Subjects			Total	
Procedure and Laboratory	N	Average Odds Favoring Carriership	Misclassification Rate (%)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	Misclassification	%
F.VIII:C Bioassay								
Milan	49	21:1	10.2	49	1:9.9	2.0	6/98	6.1
Leiden	41	46:1	7.3	16	1:18	0	3/57	5.
Malmo	41	12:1	19.5	30	1:2.8	6.7	10/71	14.0
F.VIII:Ag immunoassay								
Milan	49	11:1	22.4	49	1:5.1	4.1	13/98	13.3
Leiden	41	56:1	7.3	16	1:29	0	3/57	5.3
Malmo	41	22:1	9.8	30	1:5.9	6.7	6/71	8.4

All VWF:Ag assays were done by Laurell's electroimmunoassay method. Specific age-adjusted discriminants were calculated for each assay from each laboratory.

Table 4. Effect of Different Methods for Measuring von Willebrand Factor Antigen

		Carriers			Normal Subjects			Total	
F.VIII Assay	VWF:Ag Assay	N	Average Odds Favoring Carriership	Misclassification Rate (%)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	Misclassification	%
F.VIII:C Bioassay	EIA	49	21:1	10.2	49	1:9.9	2	6/98	6.1
	IRMA	48	18:1	8.3	49	1:8	9.1	7/97	7.1
	ELISA	48	23:1	12.5	49	1:10	10.1	9/97	9.2
F.VIII:Ag Immunoassay	EIA	49	11:1	22.4	49	1:5.1	4.1	13/98	13.3
	IRMA	48	12:1	18:8	49	1:5.2	2.0	10/98	10.2
	ELISA	48	13:1	35.4	49	1:5.9	6.1	10/98	10.2

EIA, electroimmunoassay; IRMA, immunoradiometric assay; ELISA, enzyme-linked immunosorbent assay.

All of the data originated in Milan. Separate age-adjusted discriminants were calculated for each of the six pair-wise combinations of assays.

two methods. Some laboratories are better with one assay, some with another. Each laboratory should make a choice based on its own experience.

One laboratory provided data that allowed us to examine not only two methods for assaying F.VIII:C but also three methods for assessing VWF:Ag. It was clear that bioassay of F.VIII:C was more efficient in this laboratory than was immunoassay of F.VIII:Ag, but the methods of assaying VWF:Ag did not differ appreciably; Laurell's electroimmunoassay for VWF:Ag was as satisfactory as the IRMA or the ELISA.

The ratio method for assessing potential hemophilia carriers has been widely used because of its computational simplicity. We compared the utility of linear discriminants with the utility of ratios, both as universal and as lab-specific discriminants, adjusted for age only and for age plus ABO blood type. In every instance, the linear discriminant outperformed the ratio. The ratio method not only had significantly worse average behavior but also tended to make serious misclassification errors, assigning extremely high odds for carriership to some normal subjects and vice versa.

What we have described is the analysis of a complex clinical laboratory experiment designed to determine the best methods for determining the genotypes of hemophilia A carriers from their phenotypes. Now that there are methods for determining the genotypes of carriers directly from their DNA, however, one may ask whether the phenotyping methods are obsolete. As discussed in a recent review of the advantages and limitations of phenotypic and genotypic methods,[5] there is a place for both, since there are occasions when the DNA method cannot be applied. Furthermore, even when the DNA method is applicable, the information obtained concerning genotype is often probabilistic (since the most informative polymorphic markers show a non-zero probability of crossover with the disease) and, when it is combined with information derived from the older method,

Table 5. Linear Discriminants Versus Ratio Discriminants

	Carriers			Normal Subjects			Total	
Discriminant (Adjustment)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	N	Average Odds Favoring Carriership	Misclassification Rate (%)	Misclassification	%
Linear (universal, age adjusted)*	331	11:1	16.9	136	1:4.5	8.1	67/467	14.3
Ratio (universal, age adjusted)*	331	2.4:1	17.5	136	1:1.7	16.9	81/467	17.3
Linear (universal, age, ABO)†	196	13:1	17.3	54	1:3.6	7.4	38/250	15.2
Ratio (universal, age, ABO)†	196	0.6:1	26	54	1:0.9	24.1	64/250	25.6
Linear (lab-specific, age adjusted)‡	226	22:1	14.2	136	1:8.8	2.9	36/362	9.9
Ratio (lab-specific, age adjusted)†	226	8.5:1	12.8	136	1:8.8	14.7	49/362	13.5

*Total sample.

†Subsample on which ABO data were available.

‡Subsample which consists of five laboratories providing a normal cohort.

discrimination is improved over that obtainable by either method separately.[4]

In summary, our observations have led us to make the following recommendations. First, when possible, a laboratory should endeavor to collect enough data on its local carrier population to permit the computation of its own local discriminant. When this is not possible, however, the "universal" discriminant developed here can be used as described in Appendix A, provided that a set of normal females has been assayed for F.VIII:C and VWF:Ag against the local standard and their means have been used in the adjustment procedure as described. A new group should be assayed against each new working standard.

Second, in all cases, ABO data should be collected for inclusion in the discriminant. Age data, though probably somewhat less important, should be noted as well.

Third, choice between the more traditional assays and the newer assays depends entirely on the specific experience and expertise of the laboratory. It is clear that the traditional F.VIII:C and VWF:Ag assays are fully useful in practiced hands.

Last, the ratio method of discrimination should be avoided. Its chief merit, computational simplicity, can no longer be considered important since personal computers and statistical packages are now widely available. The method described in Appendix A can be easily used on a programmable calculator.

APPENDIX

Procedure for computing probability of carriership of hemophilia A. Measurement of F.VIII:C and VWF:Ag may be done by local "in-house" methods. In using the following procedure, it is not necessary to convert from local "working" units to international units, because the adjustment procedure in step 2 will automatically correct for differences in the scale of measurement. (If a laboratory wishes its reported assay values to be comparable with those of other laboratories, it should of course calibrate its working standard against the international standard.) Assays on the normal and test subjects should be conducted under conditions as nearly identical as possible, using the same working standard. The sample on which the universal discriminant was based did not include women who were pregnant or using contraceptives; consequently, the following procedure may not be applicable to such individuals.

(1) At least 20 normal women, preferably having an age and blood group distribution comparable to those of the local population from which test subjects are drawn, should be assayed for F.VIII:C and VWF:Ag. The values should then be logarithmically transformed (using natural logarithms). Then, compute: u_x = the mean of the (log transformed) VWF:Ag values, and u_y = the mean of the (log transformed) F.VIII:C values. (2) For each individual for whom the probability of carriership is to be calculated, compute $x = \mathrm{L}n(\mathrm{VWF:Ag}) - u_x$; and $y = \mathrm{L}n(\mathrm{F.VIII:C}) - u_y$. Then compute the coefficients, a and b, of the (modified) discriminant: $a = -.0955 - .0156(\mathrm{age}) + .000196(\mathrm{age}^2) + [+.0298]$; and $b = .649 - .00184(\mathrm{age}) + .0000314(\mathrm{age}^2) + [+.117]$, and the predicted means of the discriminant for carriers and normal subjects: $c = -.391 - .00571(\mathrm{age}) + .0001(\mathrm{age}^2) + [-.0648]$; and $d = -.0347 - .00171(\mathrm{age}) + .0000473(\mathrm{age}^2) + [+.0754]$, where age is measured in years, and the final term in each line in square brackets is included if the individual does not have blood type O. (3) Compute: $e = ax + by$ (the discriminant, slightly modified); $f = 4.28\ (e - c)$; $g = 7.97\ (e - d)$; and $h = .623 + .5\ (f + g)(f - g)$. (4) Compute $R = \exp(h)$. (This is the reciprocal of the odds for carriership.)

Suppose, on the basis of data independent of the assays—pedigree data, linkage data, etc.—that the prior probability of the individual being a carrier is q. (Methods for computing q are given in[14,19] and references cited therein; a more recent example, illustrating how to incorporate data from a linked DNA marker, appears in ref. 20. As is pointed out in our accompanying article,[6] it is not generally appreciated that all available information should be used in computing q. For certain complex pedigrees, it may be necessary to use the formula below iteratively, first obtaining the probability of carriership for the maternal ancestors of the counselee, and then using this to calculate q and P for the counselee herself.) The final probability of being a carrier is then $P = q/(q + (1 - q)R)$. The above computations may easily be implemented on a programmable calculator.

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REFERENCES

1. Zimmerman TS, Ratnoff OD, Littell AS: Detection of carriers of classic hemophilia using an immunologic assay for antihemophilic factor (factor VIII). J Clin Invest 50:255, 1971

2. Elston RC, Graham JB, Miller CH, Reisner HM, Bouma BN: Probabilistic classification of hemophilia A carriers by discriminant analysis. Thromb Res 8:683, 1976

3. Wahlberg T, Blomback M, Brodin U: Carriers and noncarriers of haemophilia A: I. Multivariate analysis of pedigree data, blood coagulation tests and factor VIII variables. Thromb Res 25:401, 1982

4. Oberle I, Camerino G, Heilig R, Grunebaum L, Cazenave J-P, Crapanzano C, Mannucci PM, Mandel J-L: Genetic screening for hemophilia A (classic hemophilia) with a polymorphic DNA probe. N Engl J Med 312:682, 1985

5. Graham JB, Green PP, McGraw RA, Davis LM: Carrier detection in the hemophilias. Some limitations of the DNA methods. Blood 66:759, 1985

6. Graham JB, Rizza CR, Chediak J, Manucci PM, Briet E, Ljung R, Kasper CK, Essien EM, Green PP: Carrier detection in hemophilia "A," a co-operative international study, I: The carrier phenotype. Blood (this issue)

7. Winter RM, Tuddenham EGD, Goldman E, Matthews KB: A maximum likelihood estimate of the sex ratio of mutation rates in hemophilia A. Hum Genet 64:156, 1983

8. Orstavik KH, Magnus P, Reisner HM, Berg K, Graham JB, Nance W: Factor VIII and factor IX in a twin population. Evidence for a major effect of ABO locus on factor VIII level. Am J Human Genet 37:89, 1985

9. Peake IR, Newcombe RG, Davies BL, Furlong RA, Ludlam CA, Bloom AL: Carrier detection in haemophilia A by immunologi-

cal measurement of factor VIII related antigen (VIIIRAg) and factor VIII clotting antigen (VIIICAg). Br J Haematol 48:651, 1981

10. McCallum CJ, Peake IR, Newcombe RG, Bloom AL: Factor VIII levels and blood group antigens. Thromb Haemost 50:757, 1983

11. Mibashan RS, Peake IR, Newcombe RG, Thumpston JK, Gorer R, Furlong RA, Rodeck CH: Carrier detection of haemophilia A in pregnancy by measurement of VIII:C/RAg and VIII:CAg/RAg ratios. Thromb Haemost 46:187, 1981

12. Ratnoff OD, Jones PK: The laboratory diagnosis of the carrier state for classic hemophilia. Ann Intern Med 86:521, 1977

13. Graham JB, Flyer P, Elston RC, Kasper CK: Statistical study of genotype assignment (carrier detection) in hemophilia B. Thromb Res 15:69, 1979

14. Akhmeteli MA, Aledort L, Alexaniants S, Bulanov AG, Elston RC, Ginter EK, Goussev A, Graham JB, Hermans J, Larrieu MJ, Lothe F, McLaren AD, Manucci PM, Prentice CRM, Veltkamp JJ: Methods for detection of hemophilia carriers: A memorandum. Bull WHO 55:675, 1977

15. Lachenbruch PA: Estimation of error rates in discriminant analysis. PhD dissertation, University of California at Los Angeles, 1965

16. Lachenbruch PA: Discriminant Analysis. New York, Hafner, 1975

17. Graham J, Barrow E, Elston R: Lyonization in hemophilia: A cause of error in direct detection of heterozygous carriers. Ann NY Acad Sci 240:141, 1975

18. Hoyer LW, Carta CA, Golbus MS, Hobbins JC, Mahoney MJ: Prenatal diagnosis of classic hemophilia (hemophilia A) by immunoradiometric assays. Blood 65:1312, 1985

19. Murphy EA, Mutalik GS: The application of Bayesian methods in genetic counseling. Hum Hered 19:126, 1969

20. Kingston HM, Sarfarazi M, Newcombe RG, Willis N, Harper PS: Carrier detection in Becker muscular dystrophy using creatine kinase estimation and DNA analysis. Clin Genet 27:383, 1985

Defective Platelet-Fibrinogen Interaction in Hereditary Canine Thrombopathia

By James L. Catalfamo, Sharon L. Raymond, James G. White, and W. Jean Dodds

A unique, intrinsic, hereditary canine platelet disorder attributable to abnormal fibrinogen receptor availability is described. Thrombopathic platelets from 13 severely affected basset hounds failed to aggregate in response to all agonists tested except thrombin. Normal platelet interaction with the various stimuli was inferred on the basis of their ability to elicit unimpaired shape change in thrombopathic platelets. No quantitative differences in major platelet membrane glycoproteins, intraplatelet fibrinogen, adenine nucleotides, or serotonin uptake were detected. Dense granule secretion was impaired. The ultrastructural appearance of thrombopathic platelets was normal. Fibrinogen-platelet interaction was evaluated by reacting platelet-rich plasma (PRP) with fibrinogen coupled to polymeric acrylonitrile beads and scoring the extent of stimulus-induced agglutination. The aggregatory responses of normal and thrombopathic platelets were closely correlated with fibrinogen receptor availability. In contrast to human platelets, epinephrine-stimulated canine platelets did not interact with immobilized fibrinogen, and arachidonate generally induced only weak agglutination. Thrombopathic platelets agglutinated fibrinogen beads at reduced rates when stimulated with physiologic doses of thrombin and high-dose calcium ionophore, A23187. Our data suggest that thrombin-mediated induction of canine platelet fibrinogen receptors may proceed by pathway(s) alternate to those shared by other platelet agonists, and/or that secreted granule constituents may act synergistically with thrombin to overcome inhibition of signal-response-coupled reactions mediating the interaction of fibrinogen with its receptor. This congenital platelet defect provides further evidence, in a species other than human, for the pivotal role of fibrinogen receptor induction in platelet aggregation.

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FIBRINOGEN appears to play a central role in platelet aggregation. The requirement for plasma-derived fibrinogen in ADP-mediated aggregation is well established,[1-3] and there is evidence to suggest that platelet-derived fibrinogen also supports aggregation.[4] Several groups have demonstrated that under the appropriate physiologic conditions, specific and saturable receptors for the molecule are exposed on the platelet surface.[5-13] A good correlation between fibrinogen binding and platelet aggregability[9] but not shape change[14] exists. The surface receptor for fibrinogen is induced by ADP,[5-8] epinephrine,[6,8] collagen,[13] arachidonic acid,[13,15] or thrombin.[16] Prostaglandin I_2 has been reported to inhibit receptor exposure,[16] presumably in response to elevated levels of cyclic AMP.[17]

The putative receptor for fibrinogen, present on the surface of unstimulated platelets,[18-20] is a calcium-dependent[21-23] heterodimer complex formed by the association of two major platelet membrane glycoproteins, GPIIb and GPIIIa.[24,25] When platelets are stimulated, presumed changes in the conformation and/or microenvironment of the complex occur, through as yet undefined mechanism(s), leading to expression of specific binding sites for fibrinogen and other adhesive proteins including fibronectin, von Willebrand factor, and thrombospondin.[26]

The functional significance of the GPIIb-IIIa complex is underscored in the rare congenital bleeding disorder, Glanzmann's thrombasthenia, in which the platelets fail to aggregate in response to all known physiological stimuli.[27] This severe platelet dysfunction is attributed to the absence or marked reduction of both membrane glycoproteins[28-31] and the consequent failure of thrombasthenic platelets to bind fibrinogen.[7-9] A hereditary platelet defect having some features in common with human thrombasthenia is quite prevalent in basset hounds.[32,33] However, the specific defect in canine thrombopathia appears to be unique and may represent a platelet disorder as yet unrecognized in humans.

This article is the first detailed characterization of canine thrombopathia. Our results suggest that the severe platelet dysfunction may be due to the failure of select platelet agonists to initiate expression of sites on the platelet surface capable of normal interaction with fibrinogen. As such, it would be the first report of a congenital hereditary platelet defect attributable to abnormal fibrinogen receptor induction.

MATERIALS AND METHODS

Reagents. Sodium salts of ADP and ATP, type I bovine collagen, hirudin, (−)epinephrine bitartrate, imipramine-HCl, *N*-2-hydroxy-ethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), phosphoenolpyruvate, apyrase, *n*-ethylmalamide, orosomucoid, Triton X-114 (Rohm and Haas, Philadelphia) and pyruvate kinase were purchased from Sigma Chemical Co, St. Louis. Purified bovine albumin (Pentex fraction V) was purchased from Miles Laboratories, Elkhart, Ind; calcium ionophore, A23187, from Cal Biochem, Behring Corp, LaJolla, Calif; sodium arachidonate (SA; 99%) from Nu Check Prep, Elysian, Minn; human fibrinogen, grade L, from AB Kabi, Stockholm, Sweden; rabbit immunoglobulin to human fibrinogen from Dako, Denmark, [^{14}C]-serotonin (as 5-hydroxytryptamine creatinine sulfate, 53 mCi/mmol) from Amersham Corporation, Arlington Heights, Ill; Sepharose 4B from Pharmacia Fine Chemicals, Sweden; and polymerized acrylonitrile beads, 1 to 3 μm in diameter, from Amicon Corporation, Lexington, Mass. Highly purified human α- and γ-thrombins were generous gifts from Dr John W. Fenton II, New York State Department of Health. All other reagents were of the highest purity available.

A fibrillar suspension of bovine collagen was prepared as described previously.[34] ADP and epinephrine bitartrate were dis-

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solved in 0.15 mol/L of NaCl; SA was dissolved in 0.05 mol/L Tris–0.15 mol/L NaCl, pH 7.4, and diluted prior to use. SA solutions appeared cloudy even after dilution. α-Thrombins and γ-thrombins were diluted with gel filtered platelet elution buffer containing albumin (0.35%). [^{14}C]-serotonin was dissolved in 70% ethanol to a final activity of 8.8 μCi/mL. Ionophore A23187 was dissolved in Me_2SO. All solutions were kept frozen at −60 °C until used. Epinephrine bitartrate was freshly prepared.

Animals. The study population consisted of 16 purebred basset hounds referred to our kennel facility because of their congenital bleeding diathesis.[33] Thirteen of the dogs (six males, seven females) were clinically severely affected with thrombopathia; another 3 were asymptomatic and had an intermediate level of platelet dysfunction. The latter group were presumed to be heterozygous for the defect by virtue of having an affected parent or having produced one or more affected offspring. Some of the affected dogs were either littermates or closely related; others were more distantly related or appeared unrelated. Because the inheritance pattern of the defect within these inbred and linebred families of basset hounds is unclear at present,[33] the clinically affected animals could be either homozygous for the trait or severely penetrant heterozygotes. Mortality is high in some litters within affected bloodlines, so homozygosity may be lethal, as seen in some breeds with canine von Willebrand's disease.[33]

The 16 basset hounds and the 30 healthy dogs of various breeds, including basset hounds or mongrels that served as controls for the study, were young adults of both sexes, housed in a kennel environment. None of the bitches were in estrus or pregnant when studied, and all animals received the same diet of maintenance dog meal supplemented with raw meat; water was provided ad libitum. None of the animals had received any medication within at least 2 weeks prior to study. Data for the three putative heterozygotes are not reported here because there were too few such animals for detailed studies of their platelet function.

Platelets. Blood was drawn from the cephalic vein of the adult fasting normal or thrombopathic dogs into plastic syringes containing $^1/_{10}$ final volume of 3.8% trisodium citrate, acid citrate dextrose (4.5% Na citrate, 2.73% citric acid, 2% glucose) or 54 mmol/L of EDTA in 0.15 mol/L of NaCl. Platelet-rich plasma (PRP) was prepared at room temperature by centrifuging the blood for three 2-minute intervals at 590 g; platelet recovery exceeded 88%. For aggregation studies, the resulting supernatants were pooled and adjusted with autologous platelet-poor plasma (PPP) to a platelet count of 300,000/μ L. PPP and platelet-free plasma (PFP) were prepared at room temperature by centrifugation at 2,200 g for 15 minutes and at 16,000 g for 10 minutes, respectively.

Gel-filtered platelets (GFP) were prepared from citrated PRP (3 to 5 mL; 7.0 × 10^8 platelets per milliliter) essentially as described by Tangen and colleagues[35] on a column (1.5 × 30 cm) packed with acetone-washed Sepharose 4B and equilibrated with modified Tangen-HEPES buffer[36] (147 mmol/L of NaCl, 5 mmol/L KCl, 0.05 mmol/L of $CaCl_2$, 0.1 mmol/L of $MgCl_2$, 5 mmol/L of HEPES, 5.5 mmol/L of glucose, pH 7.4) containing 0.35% bovine albumin. Platelet recovery was >90%. GFP were diluted with elution buffer to a final count of 300,000/μL. When required, the platelet suspension was made 1 mmol/L with respect to $CaCl_2$. In some experiments, platelets were gel-filtered in divalent cation free-Tyrodes albumin (0.35%) containing 5 U/per milliliter of heparin. Washed platelets were prepared from citrated PRP by the Stractan gradient technique of Corash and colleagues.[37] For electrophoretic studies, platelets were washed three times and obtained as described by Nurden and co-workers.[38]

Platelet function. Platelet aggregation was measured turbidimetrically[39] in a dual-channel aggregometer (Payton Associates, Buffalo, NY) equipped with an OmniScribe recorder (Houston Instruments, Austin, Tex). PPP or platelet suspension buffer was used to represent 100% aggregation. In a typical experiment, 0.45 mL of PRP (300,000/μL) was pipetted into each 1-mL siliconized glass cuvette and warmed to 37 °C for one minute with stirring (850 rpm); then 0.05 mL of the appropriate agonist was added. When Me_2SO was the agonist solvent, not more than 5 μL was added to the platelet suspension. The extent of platelet aggregation was measured after 3 minutes. When dual stimuli were used, they were applied one minute apart.

Formation of microaggregates in response to extremely high doses of ADP (>150 μmol/L) was confirmed by phase-contrast microscopy. Shape change events were monitored photometrically and verified by electron microscopy. [^{14}C]-Serotonin was used as a marker for release of dense granule constituents, and release was measured essentially as described by Jerushalmy and Zucker,[40] with inclusion of a formalin fixation step prior to centrifugation to minimize reported[41] secretion artifacts associated with rapid centrifugation of A23187-treated platelets. Imipramine (3–10 μmol/L) was also included to inhibit serotonin reuptake.[42]

Retention of canine platelets on glass bead surfaces was measured by using whole blood as previously described.[43] The average number of platelets eluted in the fourth and fifth milliliter of blood was used to calculate percentage of retention. Collagen-platelet interaction was evaluated by using noncovalently immobilized collagen.[34] The ability of thrombopathic platelets to support normal clot retraction was determined according to the method of Taylor and Muller-Eberhard.[44]

Electron microscopy. For ultrastructural studies, citrated PRP (300,000/μL) was warmed with stirring (850 rpm) for 1 minute in an aggregometer; then 50 μL of the appropriate agonist or vehicle was introduced. The samples were fixed by rapidly adding one part 0.1% glutaraldehyde to the cuvette when the point of maximal optical density (platelet shape change) was reached. After ten minutes, the platelets were pelleted (800 g for five minutes); the supernatant was decanted; 1 mL of 3% glutaraldehyde was added; and the samples were sent to Dr James G. White, University of Minnesota, for processing.

Adenine nucleotides. ADP and ATP were quantitated in ethanolic extracts[45] of EDTA PRP by the firefly luciferase method of Holmsen et al[46] using an Aminco microphotometer (American Instruments Co, Silver Springs, Md) interfaced with a digital integrator.

Platelet fibrinogen. The amount of fibrinogen in normal and thrombopathic platelets was quantitated by rocket immunoelectrophoresis.[47] The procedure of Nurden[48] was adapted to measure canine platelet fibrinogen. In brief, washed normal and thrombopathic platelets (2 × 10^9 cells) were resuspended in 200 μL 0.038 mol/L of Tris and 0.1 mol/L of glycine, pH 8.6, containing 1% (vol/vol) Triton X-100 and extracted for 30 minutes at 4 °C. Triton insoluble material was removed by centrifugation. The extracts were then electrophoresed as detailed in Fig 1. The assay was standardized using canine fibrinogen. Peak heights for fibrinogen standards were linear with respect to fibrinogen concentration from 50 to 500 ng per well. Assays were performed in duplicate. A standard curve was constructed from which platelet fibrinogen levels were determined. Maximal rocket sharpness using canine antigen with rabbit anti-human fibrinogen immunoglobulins could be achieved only when the plate temperature was maintained at 28 °C during electrophoresis. Paired normal and thrombopathic platelet extracts were run on the same plates.

Fibrinogen receptor interaction. Canine fibrinogen was isolated from ACD plasma as described by Regoeczi and Stannard[49] and was further purified[50] to remove possible traces of plasminogen, fibronectin, and factors VIII and XIII. Highly purified canine fibrinogen, judged to be free of trace contaminants by sensitive and specific methods described elsewhere,[50] was 95% thrombin-clottable. When

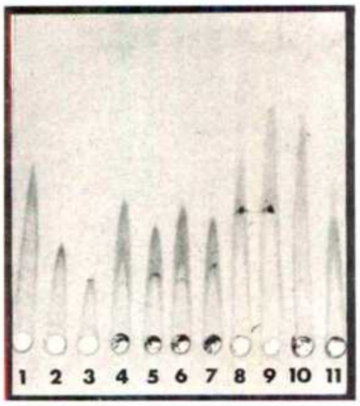

Fig 1. Quantitation of intraplatelet fibrinogen by rocket immunoelectrophoresis. Triton soluble platelet proteins were diluted at least fourfold with Triton-free extraction buffer prior to well application to minimize Triton staining artifacts (dark spots in wells 8 and 9), then electrophoresed at 10 mA per plate, overnight at 25 °C into a 0.7% agarose gel containing 62 μL rabbit anti-human fibrinogen immunoglobulins. Samples 8 μL in volume were added to each well. Contained in wells were: (1 through 3), 500, 250, and 125 ng purified canine fibrinogen; (4, 6) Triton-soluble platelet proteins from control normal dog 1 and dog 2; (5, 7, 11) platelet extracts from three thrombopathic dogs; and (8 through 10) the previous thrombopathic platelet extracts to which 200 ng of purified canine fibrinogen was added.

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the preparation was homogeneous under nonreduced conditions. When reduced, it showed the presence of intact Aα, Bβ, and γ chains.

The capacity of normal and thrombopathic canine platelets to interact with immobilized fibrinogen was assessed by the bead agglutination technique of Coller.[50] For most studies, highly purified canine fibrinogen was immobilized to polymeric acrylonitrile beads. In preliminary studies, crude (Kabi) or purified human fibrinogen was used. Beads to which BSA was similarly coupled served as controls. The extent of agglutination after platelet exposure to a stimulus was scored at three minutes, and with some agonists, at 15 seconds on a scale of 0 to 4+. A slight grainy reaction was considered 1+; a 4+ value was assigned to samples in which the beads were in relatively few but large clumps. Canine platelet interaction with bead-immobilized human fibrinogen was similar to that observed for canine fibrinogen.

Platelet glycoproteins. Washed normal or thrombopathic platelets were resuspended, solubilized, and reduced (5% vol/vol 2-mercaptoethanol) as described for human platelets.[48] Nonreduced and reduced samples containing 100 to 400 μg of protein were analyzed on 6% or 7% polyacrylamide slab gels, overlaid with a 3.5% stacking gel and electrophoresed according to Laemmli,[51] and fixed, washed, and stained with periodic-acid Schiff (PAS) reagent[52] as detailed by Clemetson.[53] Orosomucoid, 5 μg per sample, was used as an internal glycoprotein standard. Gels were scanned at 550 nanometers using a model 2400 Gilford spectrophotometer. Some gels were counterstained with Coomassie brilliant blue R 250.

Washed normal human, canine and thrombopathic platelets were also solubilized and phase extracted with Triton X-114[54] to obtain detergent phase fractions selectively enriched for integral membrane proteins (predominantly IIb and IIIa). Reduced and nonreduced SDS-solubilized detergent phase proteins, 25 to 100 μg per lane, were electrophoresed, stained (PAS), and scanned under identical conditions and often on the same slab gel as SDS-solubilized platelets.

RESULTS

Aggregation. Aggregation profiles typical for severe hereditary canine thrombopathia are presented in Fig 2. Stimulation of thrombopathic platelets with physiologic concentrations of ADP, collagen, or SA failed to induce aggregation. Unlike human platelets, canine platelet sensitivity to exogenous arachidonate was variable, as others have pre-

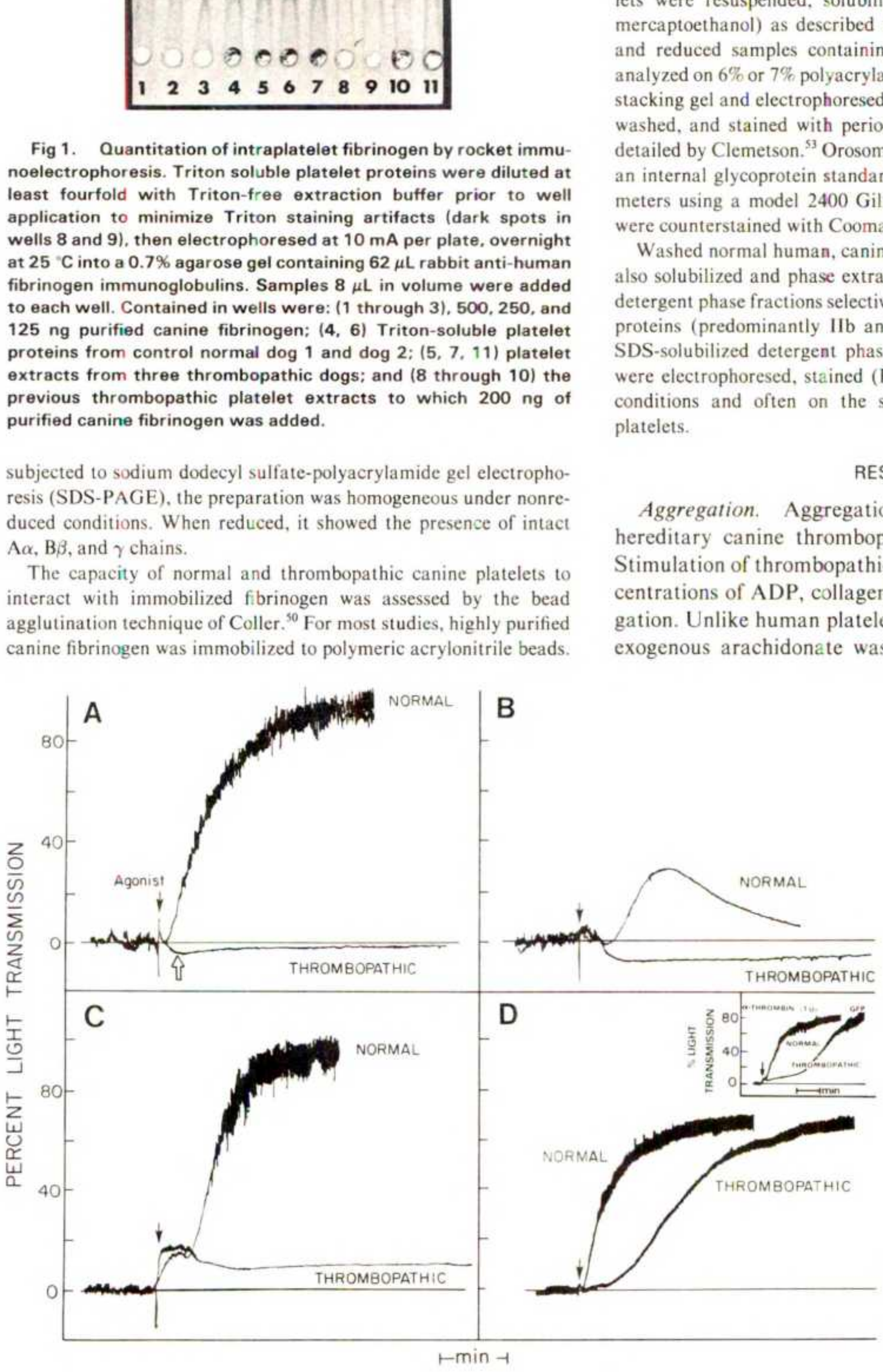

Fig 2. Typical aggregation profiles obtained for normal (control) and thrombopathic canine platelets in response to (A) ADP, (B) sodium arachidonate, (C) collagen, or (D) γ-thrombin. Aggregation is expressed as a function of increasing light transmission. Platelet activation was initated by adding 50 μL of agonist (↓) to citrated platelet-rich-plasma (300,000/μL). Platelet response to α-thrombin was evaluated in gel-filtered platelets (300,000/μL) without added fibrinogen (inset). The tracings shown are from a single experiment and are representative of results obtained for each severely affected dog (N = 13) and an equal number of controls. Occasional microaggregate formation in response to high-dose ADP (>50 μmol/L) was observed.

viously reported.[55-57] Normal basset hound platelets (N = 15) responded to SA with consistent weak reversible aggregation. Heparinized (10 U per milliliter) PRP was used in a few experiments; the aggregation profiles obtained for all agonists were similar to those shown for the citrated system.

In marked contrast to the other physiologic stimuli, thrombin induced aggregation of thrombopathic platelets. When quantitated with GFP, thrombopathic platelets aggregated to the same maximal extent as their normal counterparts over a wide range of thrombin concentrations (Fig 3A). However, the rate of the response was impaired (Fig 3C). In heparinized-GFP, no significant differences in the rate of α-thrombin-induced aggregation were observed. This may be due to the reported proaggregatory effects of heparin.[58-59] Bell and co-workers and Patterson and co-workers[60,61] have reported that thrombopathic platelets fail to aggregate in response to thrombin; however, they used a single dose of thrombin (0.2 U/per milliliter PRP).

In PRP, stimulation of thrombopathic platelets with high-dose calcium ionophore A23187 failed to induce aggregation (Fig 4). In GFP made 1 mmol/L with respect to $CaCl_2$ (Fig 4; Inset) aggregation occurred but only in response to ionophore doses eight- to tenfold higher than required by normal platelets (Fig 3B). Likewise, the rate of aggregation was markedly impaired (Fig 3D).

In other experiments, increasing the concentration of extraplatelet ionic calcium had no demonstrable effect on the reactivity of thrombopathic platelets, even in response to agonist concentrations 20- to 50-fold higher than those shown. Likewise, the severe platelet dysfunction could not be corrected by resuspending Stractan-washed platelets for up to two hours in normal canine plasma. When equal volumes of normal and thrombopathic PRP (300,000 platelets per microliter) were mixed and incubated for up to 10 minutes at 37 °C, the presence of thrombopathic platelets had no effect

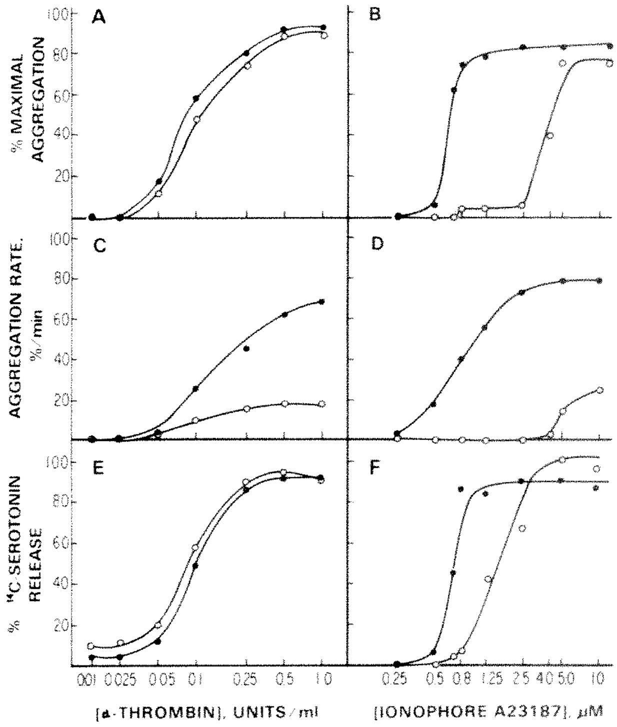

Fig 3. Dose-response curves for the maximal extent (A, B) and rate (C, D) of platelet aggregation and dense granule secretion (E, F) in normal (●) and thrombopathic (○) gel-filtered platelets induced by thrombin or calcium ionophore A23187. For each agonist, data shown are from a single experiment with determinations performed in duplicate and are representative of results obtained in five other similar experiments. GFP was made 1 mmol/L with respect to $CaCl_2$.

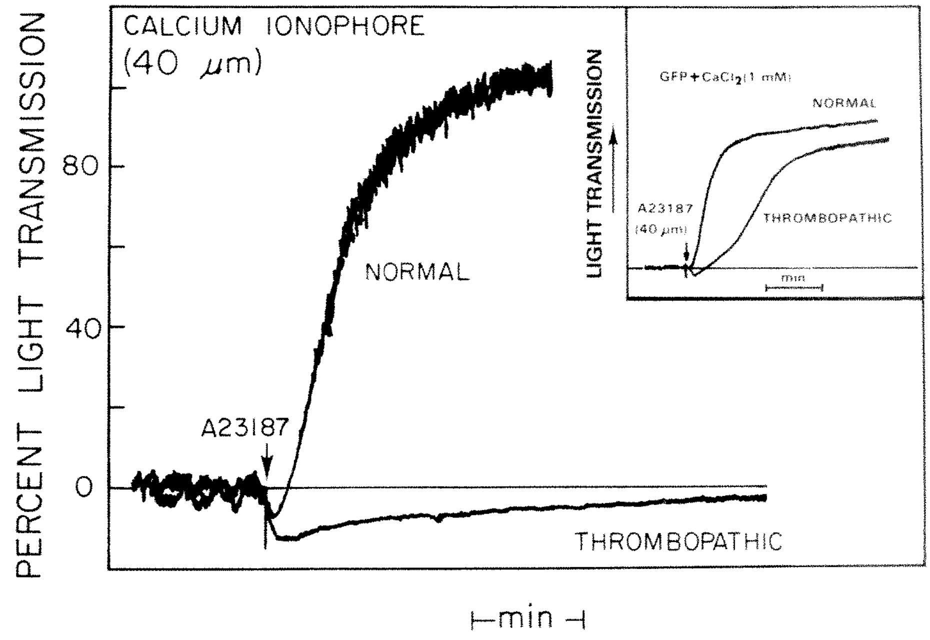

Fig 4. Aggregation response of normal (control) and thrombopathic platelets to calcium ionophore A23187. Me_2SO-solubilized ionophore (5 μL) was added to citrated platelet-rich plasma. The vehicle alone did not activate canine platelets. Irreversible aggregation of normal canine platelets could be achieved only at final ionophore concentrations >30 μmol/L. The tracings shown are from a single experiment and are typical for severe canine thrombopathia. Inset; aggregation responses typical for thrombopathic gel-filtered platelets (GFP); note impaired rate, even in the presence of 1 mmol/L of $CaCl_2$.

on the aggregatory response of normal platelets stimulated with ADP, collagen or γ-thrombin.

Shape change and morphology. Thrombopathic platelets underwent dose-dependent shape change in response to all stimuli. For all agonists examined, the onset and degree of shape change (quantitated photometrically on the basis of slope and extent of increased light scatter) paralleled those observed for normal canine platelets.

Aggregometric evidence for shape change events was confirmed by ultrastructural analysis of platelets fixed at the point of maximal light scatter (Fig 2, open arrow). Morphological changes consistent with shape change were evident upon platelet stimulation. Platelet ultrastructural reorganization in response to ADP, collagen, or thrombin was similar. The general features of thrombopathic platelet morphology appeared normal. In comparison to normal canine platelets, thrombopathic platelets appeared to be the same size and to contain the same number of dense bodies and other organelles, whose size and shape were also within normal limits.

Potentiation by epinephrine. The sensitivity of thrombopathic platelets to ADP appeared to be enhanced immediately after the animals were exposed to the mild stress of a change in kennel environment. Stress-induced activation of the sympathoadrenal system is often associated with an increase in epinephrine secretion. Because epinephrine has been proposed as a "gain controller" in canine platelet function,[62] due to its ability to increase markedly without apparent activation, platelet sensitivity to various agonists, we examined its possible potentiating effect on thrombopathic platelets. Epinephrine (5.4 μmol/L) did not activate thrombopathic platelets (Fig 5) or normal controls (not shown). It has no apparent potentiating effect on the response of thrombopathic platelets to collagen. However, preexposure to epinephrine enhanced the sensitivity of thrombopathic platelets to physiologic concentrations of ADP. Such platelets reacted with irreversible aggregation; although at rates four- to fivefold lower than those of non-epinephrine-exposed, ADP-stimulated, normal canine platelets. For thrombopathic platelets, aggregation rates approaching normal could not be achieved even when epinephrine and ADP concentrations were increased to 20 and 150 μmol/L, respectively.

Serotonin release. When the release reaction of thrombopathic platelets was evaluated in PRP (Table 1), <6% of the total platelet ^{14}C-serotonin was secreted in response to collagen (100 μg per milliliter of PRP) or calcium ionophore A23187 (40 μmol/L). Thrombin at 5 U per milliliter of PRP induced secretion exceeding 70%. ADP (8 to 10 μmol/L)-induced secretion was variable for individual thrombopathic dogs, ranging from 2% to 20%. Release was maximal at 30 seconds in contrast to platelets from normal dogs which maximally released (<6%) ^{14}C-serotonin between one to three minutes. At high ADP concentrations (68 to 100 μmol/L), secretion by thrombopathic platelets was impaired. For 12 dogs examined, the early ^{14}C-serotonin release induced by ADP represents, at its highest, less than one-third of the total secretable pool. This atypical-release response was also observed in thrombopathic platelets by Patterson and colleagues.[61,63] They suggested that thrombopathic platelets secrete the bulk of their dense granule constituents in response to 10 μmol/L of ADP and that the early release of large amounts of platelet ATP may explain why thrombopathic platelets fail to aggregate. The fact that several of the bassets (N = 5) in this study released <4% of their ^{14}C-serotonin in response to 10 μmol/L of ADP and failed to aggregate does not support this hypothesis.

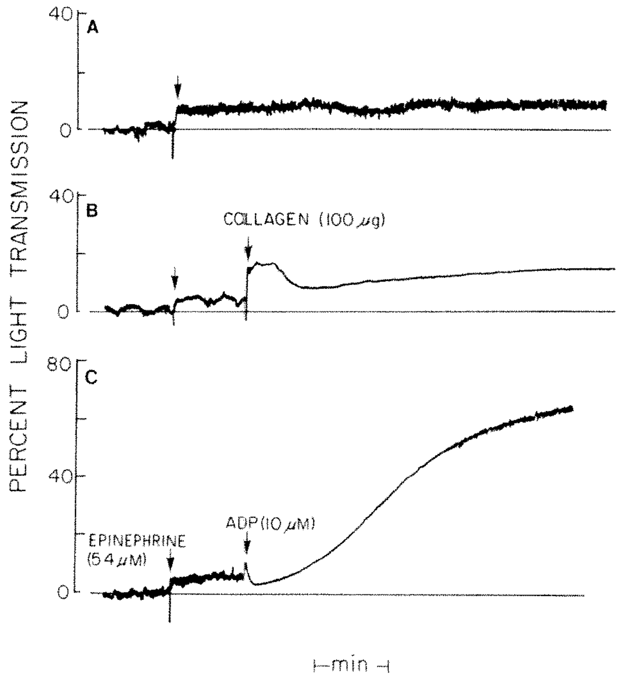

Fig 5. Epinephrine potentiation of ADP-mediated aggregation of thrombopathic platelets. (A), epinephrine; (B), epinephrine plus collagen; (C), epinephrine plus ADP.

Dose-response curves typical for ^{14}C-serotonin release by normal and thrombopathic GFP in response to α-thrombin and calcium ionophore A23187 are shown in Fig 3E and F. No significant differences in release were observed over a wide range of thrombin concentrations. In contrast, A23187 induced maximal secretion by thrombopathic platelets only

Table 1. Serotonin Release in Citrated PRP Obtained From Normal and Thrombopathic Dogs

		[^{14}C]-Serotonin Release* (%, Mean ± SD)	
Agonist	Final Concentration	Normal† N = 12	Thrombopathic N = 12
ADP	8–10 μmol/L	4 ± 2	10 ± 6
	68–100 μmol/L	51 ± 9	23 ± 11
Collagen	100 μg	66 ± 9	5 ± 1
α-Thrombin	5 U	75 ± 3	70 ± 4
γ-Thrombin	0.12 μmol/L	81 ± 9	79 ± 7
A23187	40 μmol/L	50 ± 16	5 ± 1

PRP, platelet-rich plasma.

*[^{14}C]-Serotonin uptake, after 30 minutes, 21 °C, was 92% ± 2% and 91% ± 1% for normal and thrombopathic platelets, respectively; release at three minutes.

†Canine platelets exhibit irreversible aggregation in response to doses of ADP ranging from 8 to 12 μmol/L.

at ionophore concentrations three- to fivefold higher than required by normal canine platelets.

Adenine nucleotides. The abnormal release reaction of thrombopathic platelets could not be attributed to low concentrations of platelet adenine nucleotides. No significant differences in either ADP, 1.44 ± 0.34 and 1.42 ± 0.37 μmol/10^{11} platelets or ATP, 7.07 ± 0.91 and 6.75 ± 45 μmol/10^{11} platelets were detected for thrombopathic (N = 8) and normal (N = 5) platelets, respectively.

Platelet fibrinogen. Total platelet protein and platelet fibrinogen levels were also determined. Intraplatelet fibrinogen, as illustrated for a typical experiment (Fig 1), was quantitated by rocket immunoelectrophoresis. No significant differences were found in total platelet protein, 1.73 ± 0.21 and 1.62 ± 0.27 mg/10^9 platelets, or platelet fibrinogen, 60 ± 12 and 56 ± 13 μg/10^9 platelets, for normal (N = 5) and thrombopathic (N = 12) platelets, respectively.

Retention, adhesion, and clot retraction. Functional parameters of the basic platelet reaction other than aggregation were also evaluated. No significant differences were observed between normal and thrombopathic dogs (N = 6) in percentage of platelet adhesion to fibrillar type I collagen, 51% ± 6% and 52% ± 5%, or their ability to support normal clot retraction, 90% ± 6% and 94% ± 3% retraction, respectively. However, platelets from thrombopathic dogs exhibited a marked impairment in their ability to react with and be retained by glass bead columns, 10% ± 10% *v* 94% ± 3% retention for normal platelets; despite normal levels (90% to 120% control) of factor VIII-related antigen.

Fibrinogen receptor interaction. The abnormal retention of thrombopathic platelets on glass-bead surfaces prompted us to assess the interaction of these platelets with canine fibrinogen. The bead agglutination technique of Coller[50] was used to evaluate receptor availability. Results obtained for platelets from arachidonate-insensitive normal or thrombopathic dogs are presented in Table 2.

Unstimulated normal platelets failed to interact with fibrinogen. Stimulation of normal platelets with 10 μmol/L of ADP, 0.1 U per milliliter of PRP α-thrombin, 32 nmol/L of γ-thrombin, 40 μmol/L of A23187, but not 5.4 μmol/L of epinephrine, induced maximal fibrinogen–platelet interaction. In contrast, thrombin and high-dose ionophore were the only agonists capable of initiating fibrinogen receptor availability in thrombopathic platelets. The rate of response (agglutination after 15 seconds) for both agonists was impaired. Stimulation with a combination of epinephrine and ADP induced receptor availability; however, the extent of agglutination was consistently less than that for normal platelets exposed to ADP alone.

Table 2. Agglutination of Canine Fibrinogen Beads by Normal and Thrombopathic PRP

	Agglutination*	
Stimulus	Normal (N = 6)	Thrombopathic (N = 7)
Saline	±	±
ADP [10 μmol/L]	4+	±
+EDTA (10 mmol/L)	+	+
+Fibrinogen (5 mg/mL)	2+	±
+Apyrase (2.0 mg/mL)	2+	0
+Epinephrine (5.4 μmol/L)	4+	3+
Epinephrine (5–20 μmol/L)	+	±
α-Thrombin (0.1 U/mL)†	4+	4+
+Apyrase (0.5 mg/mL)	4+	4+
Arachidonate (0.125 mmol/L)§	2+	±
+Epinephrine (5.4 μmol/L)	4+	±
γ-Thrombin (32 nmol/L)	(4+)	(3+)
	4+	4+
(16 nmol/L)	(3+)	(+)
	4+	4+
(8 nmol/L)	(+)	(±)
	2+	2+
A23187 (120 μmol/L)	(4+)	(+)
	4+	3+
(80 μmol/L)	(4+)	(+)
	4+	3+
(40 μmol/L)	(4+)	(±)
	4+	±

Normal dogs were arachidonate insensitive.

*Scored after three minutes at room temperature. Scores in parentheses are after 15 seconds. Maximal agglutination shown.

†GFP were reacted with immobilized fibrinogen after the thrombin was neutralized with excess hirudin. Hirudin alone did not inhibit ADP-stimulated platelet reactivity with immobilized fibrinogen.

§Required warming reaction mixture to 37 °C.

Platelet glycoproteins. Normal platelet interaction with fibrinogen requires the presence of GPIIb and GPIIIa in normal amounts. Thrombopathic platelet glycoproteins were therefore analyzed by SDS-PAGE (Fig 6). No quantitative differences in major platelet glycoproteins were detected. Under identical extraction conditions, no differences were observed in the protein content of detergent phases from human, normal canine, or thrombopathic platelets. Mobilities of PAS-staining bands in this fraction (predominantly IIB and IIIa) were identical to glycoprotein reactive bands from whole platelets. Canine PAS reactive bands designated as IIb and IIIa (Fig 6, arrows) comigrated with human IIb and IIIa and had relative mol wts (reduced and nonreduced) similar to those of their human counterparts.

DISCUSSION

Canine thrombopathia appears to be a unique, autosomally inherited platelet disorder without a recognized human counterpart. Unlike platelets from human type I Glanzmann's thrombasthenia,[27] thrombopathic platelets aggregated in response to thrombin; exhibited impaired dense granule secretion; supported normal clot retraction; and had normal amounts of PAS-reactive GPIIb-GPIIIa and intraplatelet fibrinogen. No support for the proposal[64] that thrombopathia may be a model for the even rarer type II form of Glazmann's thrombasthenia was obtained.

The failure of a wide array of platelet stimuli to induce aggregation, dense granule secretion, and fibrinogen receptor exposure suggests that the defect occurs at a locus or loci subsequent to agonist receptor occupancy. Indirect support for this is provided by the unaltered shape-change response of thrombopathic platelets. That thrombopathic membranes have normal collagen receptor density was demonstrated by our studies of platelet adhesion to immobilized collagen.

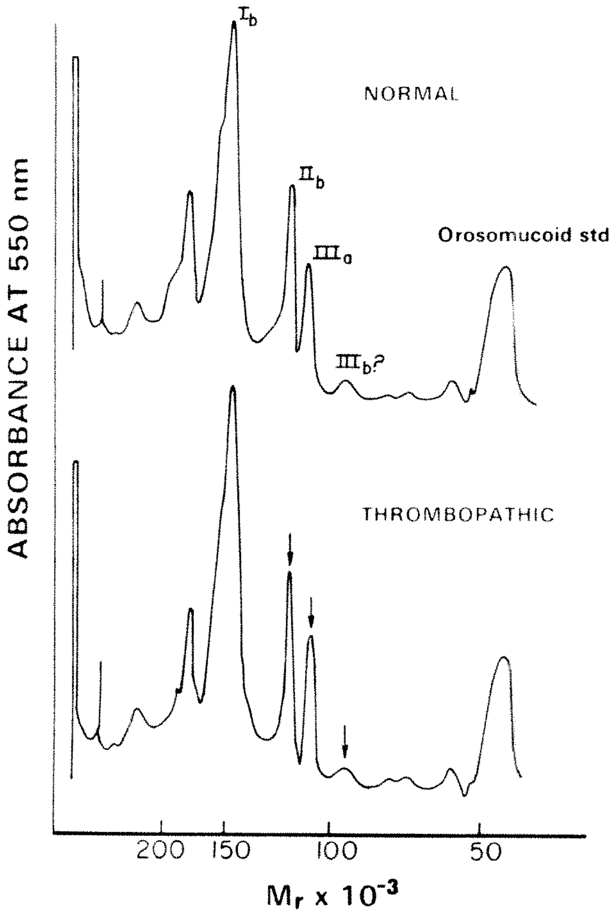

Fig 6. Densitometric scans of periodic-acid Schiff (PAS)-reactive canine platelet glycoproteins. Washed normal and thrombopathic platelets were solubilized in 2% sodium dodecyl sulfate (SDS). Immediately prior to SDS-polyacrylamide gel electrophoresis, extracts were reduced (5% vol/vol 2-mercaptoethanol) for one hour at 37 °C and electrophoresed through a 6.0% polyacrylamide resolving slab gel[48]; 400 μg of platelet protein containing 5 μg of orosomucoid standard was applied to each lane; mol wt standards were myosin (mol wt 200,000), β-galactosidase (mol wt = 116,250), phosphorylase b (mol wt = 92,500), bovine serum albumin (mol wt = 66,000), and ovalbumin (mol wt = 45,000).

Thrombopathic platelets also responded abnormally to calcium ionophore A23187, an agonist which bypasses the normal receptor process.

The concentrations of adenine nucleotides in thrombopathic platelets agreed with those reported[62] for canine platelets and are consistent with the metabolic requirements for platelet support of normal clot retraction. The possibility that canine thrombopathia is a storage-pool-like defect was considered, but the previous results, together with ultrastructural evidence of normal number and appearance of dense granules and normal serotonin uptake ruled out this classification.

A key functional abnormality associated with thrombopathia is the marked impairment of platelet retention by glass-bead surfaces, a fibrinogen-dependent process.[65–67] Because two fibrinogen-dependent reactions—aggregation and retention—were abnormal, we examined the ability of these platelets to interact with fibrinogen.

Fibrinogen-platelet interactions were evaluated using the bead agglutination technique of Coller.[50] This assay is sensitive and specific for fibrinogen receptor availability (fibrinogen binding sites).[68] Although it does not provide the detailed kinetic data concerning fibrinogen-platelet interactions obtainable using ^{125}I-radiolabeled fibrinogen, it had the advantage of allowing us to evaluate platelet-fibrinogen interaction in a large number of normal and thrombopathic dogs using an array of agonists under various experimental conditions. The essential features of normal canine platelet interaction with bead-immobilized fibrinogen were similar to those reported for human platelets.[50] Platelet stimulation was required for receptor availability. Interaction required extraplatelet divalent cations, presumably for the maintenance of GPIIb-GPIIIa complexes. In contrast to human platelets,[6,8,13,15] epinephrine-stimulated canine platelets did not interact with immobilized fibrinogen, and arachidonate induced weak agglutination. In the presence of apyrase, ADP-induced receptor expression was reduced whereas thrombin-induced exposure was unaffected. Fibrinogen binding sites were exposed on thrombopathic platelets only in response to thrombin and high-dose calcium ionophore. The possibility that the thrombin response was an artifact related to fibrin formation was considered; however, it seemed unlikely since γ-thrombin-stimulated thrombopathic platelets agglutinated bead-immobilized fibrinogen whereas γ-thrombin in the absence of platelets had no effect. For both thrombin and calcium ionophore, the rate of receptor induction was impaired, which correlated with the impaired aggregation rates seen in GFP for both agonists.

Electrophoretic analysis of thrombopathic platelets failed to demonstrate significant quantitative differences in the major platelet glycoproteins, in particular GPIIb-GPIIIa. Patterson and co-workers[63] reported similar results. They also demonstrated normal levels of the GPIIb-GPIIIa complex, thus distinguishing canine thrombopathia from a recently described variant of human Glanzmann's disease with reduced levels of the complex.[69]

Stimulation of platelets with thrombin[70] or ADP[71] causes an increase in intracellular ionized calcium, which is a prerequisite for the secretory[72] and aggregatory[71] responses of platelets. The increase appears to result from a release of Ca^{2+} from intracellular sites and influx of extracellular Ca^{2+}.[73] In addition, 1,2-diacylglycerol[74] and inositoltriphosphate[75] may serve as intracellular messengers. Thrombin and collagen induce phosphoinositide breakdown and lead to a transient increase in cellular 1,2-diacylglycerol.[74] It has been proposed that Ca^{2+} and 1,2-diacylglycerol act as coordinate second messengers in platelet activation[76] by greatly increasing the affinity of protein kinase C for Ca^{2+}.[77] Thrombin stimulation results in an increase in both internal ionized calcium and 1,2-diacylglycerol,[78] whereas collagen increases only 1,2 diacylglycerol and has no apparent effect on the concentration of intracellular ionized calcium.[76] Although Ca^{2+} acts as an activator second messenger, cAMP can serve as an inhibitory second-messenger that antagonizes the mobilization of Ca^{2+}.[79] It is also known that cAMP inhibits the hydrolysis of inositol lipids.[74,80] The impaired responsiveness of thrombopathic platelets to Ca^{2+} ionophore A23187 implies that Ca^{2+} mobilization and/or utilization may be abnormal. This is consistent with our recent observation of elevated cAMP levels in thrombopathic platelets.[81] It appears highly probable that mobilized Ca^{2+} is rapidly resequestered into intracellular storage sites. The potentiation by epinephrine of ADP-induced receptor exposure and aggregation is likely related to inhibition of adenylate cyclase in thrombopathic platelets.

The ability of thrombin and high-dose ionophore to initiate thrombopathic platelet fibrinogen receptor availability, although at impaired rates, may be due to their ability to initiate generation of coordinate second messenger(s) which serves to lower requirements for ionic calcium in reactions leading to platelet activation. The failure of thrombopathic platelets to secrete dense granule constituents and express fibrinogen binding sites appears to be related to abnormal second-messenger signal generation. Because the details of reactions leading to expression of fibrinogen binding sites on GPIIb-GPIIIa complexes have yet to be established, thrombopathic platelets may serve as a model system for the assignment of functional significance to biochemical events implicated in receptor induction.

ACKNOWLEDGMENT

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REFERENCES

1. Cross MJ: Effect of fibrinogen on the aggregation of platelets by adenosine diphosphate. Thromb Diath Haemorrh 12:524, 1964

2. Solum NO, Stormorken H: Influence of fibrinogen on the aggregation of washed human blood platelets induced by adenosine diphosphate, thrombin, collagen and adrenalin. Scand J Clin Lab Invest 17:170, 1965

3. McLean JR, Maxwell RE, Herther D: Fibrinogen and adenosine diphosphate induced aggregation of platelets. Nature 202:605, 1965

4. Tollefson DM, Majerus PW: Inhibition of human platelet aggregation by monovalent antifibrinogen antibody fragments. J Clin Invest 55:1259, 1975

5. Marguerie GA, Plow EF, Edgington TS: Human platelets possess an inducible and saturable receptor specific for fibrinogen. J Biol Chem 254:5357, 1979

6. Peerschke EI: Induction of human platelet fibrinogen receptors by epinephrine in the absence of released ADP. Blood 60:71, 1982

7. Mustard JF, Kinlough-Rathbone RL, Packham MA, Perry DW, Harfenist EJ, Pai KRM: Comparison of fibrinogen association with normal and thrombasthenic platelets on exposure to ADP or chymotrypsin. Blood 54:978, 1979

8. Bennett JS, Villaire G: Exposure of platelet receptors by ADP and epinephrine. J Clin Invest 63:1393, 1979

9. Peerschke EI, Zucker MB, Grant RA, Egan JJ, Johnson MM: Correlation between fibrinogen binding to human platelets and platelet aggregability. Blood 55:841, 1980

10. Plow EF, Marguerie GA: Induction of the fibrinogen receptor on human platelets by epinephrine and the combination of epinephrine and ADP. J Biol Chem 255:10971, 1980

11. Plow EF, Marguerie GA: Participation of ADP in the binding of fibrinogen to thrombin-stimulated platelets. Blood 56:553, 1980

12. Cierniewski CS, Kowalska MA, Krajewski T, Janiak A: Binding of fibrinogen molecules to pig platelets and their membranes. Biochem Biophys Acta 714:543, 1982

13. DiMinno G, Thiagarajan P, Perussia B, Martinez J, Shapiro S, Trinchieri G, Murphy S: Exposure of platelet fibrinogen sites by collagen, arachidonic acid and ADP: Inhibition by a monoclonal antibody to the glycoprotein IIb-IIIa complex. Blood 61:140, 1983

14. Peerschke EI, Zucker MB: Relationship of ADP-induced fibrinogen binding to platelet shape change and aggregation elucidated by use of colchicine and cytochalasin B. Thromb Haemost 43:58, 1980

15. Bennett JS, Villaire G, Burch JW: A role for prostaglandins and thromboxanes in the exposure of platelet fibrinogen receptors. J Clin Invest 68:981, 1981

16. Hawiger J, Parkinson S, Timmons S: Prostacyclin inhibits mobilization of fibrinogen-binding sites on human ADP-and thrombin-treated platelets. Nature 283:195, 1980

17. Graber SE, Hawiger J: Evidence that changes in platelet cyclic AMP levels regulate the fibrinogen receptor on human platelets. J Biol Chem 257:14606, 1982

18. McEver RP, Bennett EM, Martin MN: Identification of two structurally and functionally distinct sites of human platelet membrane glycoprotein IIb–IIIa using monoclonal antibodies. J Biol Chem 258:5269, 1983

19. Pidard D, Montgomery RR, Bennett JS, Kunicki TJ: Interaction of AP-2, a monclonal antibody specific for the human platelet glycoprotein IIb–IIIa complex, with intact platelets. J Biol Chem 258:12582, 1983

20. Hourdille P, Benabdallah S, Belloc F, Nurden AT: Distribution of glycoprotein II_b–III_a complexes in the surface membranes of human platelets and megakaryocytes. Br J Haematol 59:471, 1985

21. Fujimura K, Phillips DR: Calcium cation regulation of glycoprotein IIb–IIIa complex formation in platelet plasma membranes. J Biol Chem 258:10247, 1983

22. Gogstad GO, Hagen I, Krutsnes MB, Solum NO: Dissociation of the glycoprotein IIb–IIIa complex in isolated human platelet membranes: Dependence of pH and divalent cations. Biochim Biophys Acta 689:21, 1982

23. Kunicki TJ, Pidard D, Rosa JP, Nurden AT: The formation of Ca^{++}-dependent complexes of platelet membrane glycoproteins IIb and IIIa in solution as determined by crossed immunoelectrophoresis. Blood 58:268, 1981

24. Nachman RL, Leung LLK: Complex formation of platelet membrane glycoproteins II_b and III_a with fibrinogen. J Clin Invest 69:263, 1982

25. Bennett JS, Hoxie JA, Leitman SF, Villaire G, Cines DB: Inhibition of fibrinogen binding to stimulated human platelets by a monoclonal antibody. Proc Natl Acad Sci USA 80:2417, 1983

26. Plow EF, McEver RP, Coller BS, Woods VL, Marquerie GA, Ginsberg MH: Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor and thrombospondin on thrombin-stimulated human platelets. Blood 66:724, 1985

27. Caen J: Glanzmann's thrombasthenia. Clin Haematol 1:383, 1972

28. Nurden AT, Caen JP: An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. Br J Haematol 28:253, 1974

29. Phillips DR, Jenkins CSP, Luscher EF, Larrieu MJ: Molecu-

lar differences of exposed surface proteins on thrombasthenic platelet plasma membranes. Nature 257:599, 1975

30. Phillips DR, Agin PP: Platelet membrane defects in Glanzmann's thrombasthenia: Evidence for decreased amounts of two major glycoproteins. J Clin Invest 60:535, 1977

31. Jamieson GA, Okumura T, Fishback B, Johnson MM, Egan JJ, Weiss HJ: Platelet membrane glycoproteins in thrombasthenia, Bernard-Soulier syndrome and storage pool disease. J Lab Clin Med 93:652, 1979

32. Johnstone IB, Lotz F: An inherited platelet function defect in basset hounds. Can Vet J 20:211, 1979

33. Dodds WJ: Second International Registry of Animal Models of Thrombosis and Hemorrhagic Diseases. ILAR News 24:R18, 1981

34. Zabinski MP, Raymond SL, Catalfamo JL: Platelet adhesion to noncovalently immobilized collagen. J Lab Clin Med 103:236, 1984

35. Tangen O, Berman HJ, Marfey P: Gel filtration—A new technique for separation of blood platelets from plasma. Thromb Diath Haemorrh 25:268, 1971

36. Carroll RC, Gerrard JM: Phosphorylation of platelet actin-binding protein during platelet activation. Blood 59:466, 1982

37. Corash L, Shafer B, Perlow M: Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. Blood 52:726, 1978

38. Nurden AT, Dupuis D, Kunicki TJ, Caen JP: Analysis of the glycoprotein and protein composition of Bernard-Soulier platelets by single and two dimensional SDS-polyacrylamide gel electrophoresis. J Clin Invest 67:1431, 1981

39. Born GVR: Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 194:927, 1962

40. Jerushalmy Z, Zucker MB: Some effects of fibrinogen degradation products (FDP) on blood platelets. Thromb Diath Haemorrh 15:413, 1966

41. Holmsen H, Dangelmaier CA: Evidence that the platelet plasma membrane is impermeable to calcium and magnesium complexes of A23187. J Biol Chem 256:10449, 1981

42. Walsh PN, Gagnatelli G: Platelet antiheparin activity: Storage site and release mechanism. Blood 44:157, 1974

43. Dodds WJ, DiNovo JM, Bergeron JA: A native whole blood assay for blood-materials interaction. Thromb Haemost 45:12, 1981

44. Taylor FB Jr, Muller-Eberhard HJ: Qualitative description of factors involved in the retraction and lysis of dilute whole blood clots and in the aggregation and retraction of platelets. J Clin Invest 49:2068, 1970

45. Holmsen H, Storm E, Day HJ: Determination of ATP and ADP in blood platelets: A modification of the firefly luciferase assay for plasma. Anal Biochem 46:489, 1972

46. Holmsen H, Holmsen I, Bernhardsen A: Microdetermination of adenosine diphosphate and adenosine triphosphate in plasma with the firefly luciferase system. Anal Biochem 17:456, 1966

47. Laurell CB: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 15:45, 1966

48. Nurden AT, Kunicki TJ, Dupuis D, Soria C, Caen JP: Specific protein and glycoprotein deficiencies in platelets isolated from two patients with the gray platelet syndrome. Blood 59:709, 1982

49. Regoeczi E, Stannard BA: In vivo behaviour of frozen and freeze-dried fibrinogen and that prepared from outdated blood. Biochim Biophys Acta 181:287, 1969

50. Coller BS: Interaction of normal, thrombasthenic, and Bernard-Soulier platelets with immobilized fibrinogen: Defective platelet fibrinogen interaction in thrombasthenia. Blood 55:169, 1980

51. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1975

52. Segrest JD, Jackson RL: Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, in Ginsburg V (ed): Methods in Enzymology, Vol XXVIII. New York, Academic Press, 1972, p 54

53. Clemetson KJ, Capitanio A, Luscher EF: High resolution two-dimensional gel electrophoresis of the proteins and glycoproteins of human blood platelets and platelet membranes. Biochem Biophys Acta 553:11, 1979

54. Newman PJ, Knipp MA, Kahn RA: Extraction and identification of human platelet integral membrane proteins using Triton X-114. Thromb Res 27:221, 1982

55. Chignard M, Vargaftig BB: Dog platelets fail to aggregate when they form aggregating substances upon stimulation with arachidonic acid. Eur J Pharmacol 38:7, 1976

56. Chignard M, Vargaftig BB, Sors H, Dray F: Synthesis of thromboxane B_2 in incubates of dog platelet-rich plasma with arachidonic acid and its inhibition by different drugs. Biochem Biophys Res Commun 85:1631, 1978

57. Johnson GS, Rao GHR, Leis LA, White JG: Effect of agents that alter cyclic AMP on arachidonate-induced platelet aggregation in the dog. Blood 55:722, 1980

58. MacIntyre DE, Handin RI, Rosenberg R, Salzman EW: Heparin opposes prostanoid and non-prostanoid platelet inhibitors by direct enhancement of aggregation. Thromb Res 22:167, 1981

59. Ruggiero M, Fedi S, Bianchini P, Vannucchi S, Chiarugi V: Molecular events involved in the proaggregating effect of heparin on human platelets. Biochem Biophys Acta 802:372, 1984

60. Bell TG, Leader RW, Olson PM, Padgett GA, Penner JA, Patterson WR: Basset hound hereditary thrombopathy: An autosomally recessively inherited platelet dysfunction, in Ryder OA, Byrd ML (eds): One Medicine. New York, Springer-Verlag, 1984, p 335

61. Patterson WR, Padgett GA, Bell TG: Abnormal release of storage pool adenine nucleotides from platelets of dogs affected with basset hound hereditary thrombopathy. Thromb Res 37:61, 1985

62. Meyers KM: Pathobiology of animal platelets, in Cornelius CE, Simpson C (eds): Advances in Veterinary and Comparative Medicine, vol 30. New York, Academic Press, 1985, p 131

63. Patterson WR, Padgett GA, Bell TG: Platelet aggregation, adenine nucleotide release and membrane glycoproteins in dogs affected with basset hound hereditary thrombopathy. Thromb Haemost 54:245, 1985 (abstr)

64. Mattson JC, Estry DW, Bell TG, Patterson WR: Defective contact activation of platelets in basset hound hereditary thrombopathia. Thromb Haemost 54:188, 1985 (abstr)

65. Gugler E, Luscher EF: Platelet function in congenital afibrinogenemia. Thromb Diath Haemorrh 14:361, 1965

66. Zucker MB, Vroman L: Platelet adhesion induced by fibrinogen adsorbed onto glass. Proc Soc Exp Biol Med 131:318, 1969

67. Bergeron JA, DiNovo JM, Dodds WJ: Fibrinogen mediates platelet adhesion in bead columns from flowing whole blood. J Lab Clin Med (submitted for publication)

68. Coller BS, Peerschke EI, Scudder LE, Sullivan CA: A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds glycoproteins IIb and/or IIIa. J Clin Invest 72:325, 1983

69. Nurden AT, Rosa JP, Biozard B, Didry D, Legrand C: Evidence that GPIIb-IIIa complexes are required for ADP-induced platelet aggregation from studies on the platelets of a patient with a new type of Glanzmann's thrombasthenia. Thromb Haemost 50:216, 1983 (abstr)

70. Rink TJ, Smith SW, Tsien RY: Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. FEBS Lett 148:21, 1982

71. Owen NE, LeBreton GC: Ca^{2+} mobilization in blood platelets as visualized by chlortetracycline fluorescence. Am J Physiol 241:H631, 1981

72. Knight DF, Scrutton MC: The relationship between intracellular second messengers and platelet secretion. Biochem Soc Trans 12:969, 1984

73. White JG, Rao GHR, Gerrard JM: Effects of the ionophore A23187 on blood platelets. Am J Pathol 77:135, 1974

74. Kaibuchi K, Sano K, Hoshijima M, Taki Y, Nishizuka Y: Phosphatidylinositol turnover in platelet activation; calcium mobilization and protein phosphorylation. Cell Calcium 3:323, 1982

75. Berridge MJ, Irvine RF: Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature 312:315, 1984

76. Rink TJ, Sanchez A, Hallam TJ: Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. Nature 305:317, 1983

77. Takai Y, Kishimoto A, Kawahara Y, Minakuchi R, Sano K, Kikkawa U, Mori T, Yu B, Kaibuchi K, Nishizuka Y: Calcium and phosphatidylinositol turnover as signalling for transmembrane control of protein phosphorylation. Adv Cyclic Nucleotide Res 14:301, 1981

78. Hallam TJ, Sanchez A, Rink TJ: Stimulus-response coupling in human platelets. Changes evoked by platelet-activating factor in cytoplasmic free calcium monitored with the fluorescent calcium indicator quin 2. Biochem J 218:819, 1984

79. Zavoico GB, Feinstein MB: Cytoplasmic Ca^{2+} in platelets is controlled by cyclic AMP: Antagonism between stimulators and inhibitors of adenylate cyclase. Biochem Biophys Res Commun 120:579, 1984

80. Rittenhouse-Simmons S: Production of diglyceride from phosphatidylinositol in activated human platelets. J Clin Invest 63:580, 1979

81. Boudreaux MK, Catalfamo JL, Dodds WJ: Elevated cyclic AMP levels in canine thrombopathia. Thromb Haemost 54:278, 1985 (abstr)

Clinical and Phenotypic Diversity of T Cell Lymphomas

By Sandra J. Horning, Lawrence M. Weiss, Gertrude S. Crabtree, and Roger A. Warnke

Forty-one cases of T cell lymphoma were identified on the basis of morphology and the expression of two or more T cell antigens with an absence of B cell markers. Mycosis fungoides and lymphoblastic lymphoma were excluded. Marked clinical, morphological, and immunologic diversity was observed. Cutaneous lymphoma was found in ~50% of the patient group, and 27% had a prior history of dermatologic or immunologic disease. No correlations among immunologic and morphologic phenotypes and clinical course were apparent. Survival data was comparable to that of a concurrent group of non-T cell lymphoma patients studied at this institution, suggesting that, contrary to previous reports, T cell lymphoma may not necessarily confer a more unfavorable prognosis. Prospective studies using uniform treatments are necessary to address the clinical significance of the T cell phenotype definitively, independent of established histologic and clinical features.

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MALIGNANT LYMPHOMAS of T cell origin have been broadly classified as those expressing thymic antigens (lymphoblastic lymphoma) or postthymic, "mature" antigens. The latter group includes mycosis fungoides (MF) and Sézary syndrome, which have well-described clinical, pathologic, and immunologic features. The term "peripheral" T cell lymphoma has been applied to the heterogeneous group of diffuse lymphomas other than MF which express postthymic T cell antigens.

Varied descriptions of the morphological appearance of T cell lymphomas have been provided by pathologists in this country, Europe, and Japan.[1-12] The clinical features described for these T cell lymphomas have been nearly as heterogeneous as their morphology.[13-21] The criteria for diagnosis of T cell lymphoma have not been uniform but have usually included the identification of neoplastic cells within E rosettes. Expert pathologists are not able to distinguish between B and T cell lymphomas by morphology alone, even in the most distinctive entities.[22]

We have previously reported on the clinical relevance of immunophenotype in diffuse large cell lymphoma, which included a small number with the T cell phenotype.[23] The current study was undertaken to expand these observations and correlate the clinical characteristics with morphological and immunologic features of diffuse lymphomas expressing T cell antigens.

MATERIALS AND METHODS

Sixty-four cases of T cell lymphoma (TCL), excluding MF and lymphoblastic lymphoma, were identified in the Laboratory of Immunopathology at Stanford University Medical Center from 1981 through 1984. Further review excluded 13 cases from this analysis due to inadequate material or reinterpretation of histologic or immunologic features. Clinical data was not available for correlation in nine cases; one case was excluded because malignant lymphoma developed in association with the Wiskott-Aldrich syndrome. Thus, 41 cases of T cell lymphoma, identified on the basis of morphology and the expression of two or more T cell antigens with an absence of B cell markers, are studied in this report. Clinicopathologic correlations in ten of these caes have been previously reported.[23] In addition, a detailed morphological and immunohistochemical characterization of 39 of the 41 cases has been recently reported.[24] No patient was excluded from this series based on clinical behavior.

Tissue samples were obtained from the following sites: lymph node (19 cases), skin (17 cases), lung (2 cases), nasopharynx (2 cases), and one case each in spleen, gastrointestinal tract, mediastinum, and chest wall. These samples included initial diagnostic material in 31 cases, disease recurrent after therapy in 7 cases, and both initial and recurrent material in 3 cases. The hematoxylin-stained and eosin-stained paraffin sections of all cases were classified according to a modification of the Working Formulation for the non-Hodgkin's lymphomas.[8] Angioimmunoblastic lymphadenopathy-like ("AILD-like") T cell lymphoma as morphologically defined by Shimoyama was included as a histologic subcategory of diffuse, mixed small and large cell lymphoma.[12] A new category was also added, that of monomorphic medium-sized lymphoma as defined by Japanese pathologists.[11]

Immunologic phenotyping was performed as previously described.[25] The monoclonal antibodies detailed in Table 1 were used as a first stage incubation. After washing, either biotin-conjugated $F(ab')_2$ goat anti-mouse antibody (Tago, Burlingame, Calif), or horse anti-mouse antibody (Vector Labs, Inc, Burlingame) was applied followed by avidin-conjugated horseradish peroxidase (Vector Labs, Inc) as a third stage. Tissue sections were then incubated in diaminobenzidine followed by copper sulfate and counterstained with methylene blue. In addition to the monoclonal antibodies reactive with our T cell lymphomas listed in Table 1, monoclonal antibodies to μ heavy chains, κ and λ light chains, and the B lineage markers B1 or T105 were applied with negative results.

Clinical data were extracted and actuarial survival curves were generated according to the method of Kaplan and Meier.[26] Survival was calculated from the time of diagnosis. Tests of statistical significance between survival curves were calculated as described by Gehan.[27] Staging followed the convention of the Ann Arbor Conference and was based on results of chest x-ray, lymphography and/or computerized tomography of the abdomen, and pelvis and bone marrow biopsy, in addition to physical examination.[28] However, original staging data was not available for review in eight patients and three patients did not have bone marrow biopsies. Thirty-seven patients were primarily managed or seen in consultation by the clinical or academic Stanford faculty. The clinical records were obtained from the responsible physician in the remaining four cases.

RESULTS

Clinical and morphological features. Table 2 shows the clinical and morphological features of the study. The study included 19 females and 22 males. Ages ranged from 4 to 91

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Table 1. Monoclonal Antibodies Reactive With TCL

Antigen/Clone/Similar Antigens	Expression
Leu 1/L17F12*/T1,T65	T cells, thymocytes, B-CLL, B lymphoma
Leu 2a/SK1*/T5,T8	Cytotoxic/suppressor T cells, thymocytes, splenic sinusoids
Leu 3a/SK3*/T4	Helper T cells, thymocytes, monocytes, macrophages, Langerhan's cells
Leu 4/SK7*/T3	T cells, thymocytes, Purkinje cells
Leu 5/ATM1.1*/T11,Lyt 3	T cells, thymocytes
Leu 6*/SK9/T6	Cortical thymocytes, Langerhan's cells
Leu 9/4H9†/3A1	Cytotoxic/suppressor T cells, helper T cells, thymocytes, monocytes, macrophages
Ia/L203†/L227†	Activated T cells, B cells, myeloids, monocytes, macrophages, Langerhan's cells, epithelium

*Obtained from Becton Dickinson, Mountain View, Calif.
†Obtained from Ronald Levy Laboratory, Stanford, Calif.

Table 2. Summary of Clinical and Morphological Features in 41 Patients With TCL

Characteristic	No. of Patients	%
Stage I and II(E*)	14(9)	34(22)
Stage III and IV	27	66
Cutaneous lymphoma (only†)	20(10)	49(24)
Prior history of dermatologic or immunologic disease	11	27
Morphologic diagnosis‡		
Diffuse mixed (AILD-like)	8(3)	20
Diffuse large cell	12	30
Diffuse large cell, immunoblastic	14	34
Monomorphic	7	17
Primary Therapy		
Chemotherapy	24	59
Radiotherapy	11	27
Combined modality	3	6
None	2	5
Surgery	1	2

*Extranodal site.
†Lymphoma confined to skin.
‡Described in text.

years with a median of 56 years. Limited disease was observed in 13 patients (11 with stage I and two with stage II), nine of whom had lymphoma in extranodal sites. The sites of extranodal involvement in these limited-stage patients consisted of skin in seven cases and nasopharynx and lung in one each. Twelve patients had stage III and 16 patients had stage IV disease. Systemic symptoms were present at diagnosis in 11 patients (27%), all of whom had advanced disease. Extranodal sites of lymphoma in the entire group of 41 patients included skin (20 cases), bone marrow (4 cases), liver (4 cases), lung (5 cases), gastrointestinal tract (2 cases), nasopharynx (2 cases), and one case each involving the heart and pleura. In two patients, subsequent bone marrow biopsies, sampled following three and six cycles of chemotherapy, showed no evidence for lymphoma but were markedly hyperplastic with prominent myelodysplastic features. One of these patients had a peripheral eosinophilia of 59% (absolute count 5,900/μL). Peripheral eosinophilia was noted in one additional patient at diagnosis.

Eleven patients (27%) had a prior history of dermatologic or immunologic disease including: psoriasis (2 cases), chronic exfoliative dermatitis (2 cases), celiac sprue (2 cases), and one case each with a history of Sjogren's syndrome, rheumatoid arthritis with iritis, erythema multiforme, Sweet's syndrome, immune thrombocytopenia with auto-antibodies and nonspecific dermatitis. Cutaneous lymphoma was present in six of these 11 patients.

Of the 20 patients with cutaneous lymphoma at diagnosis, disease was restricted to the skin in ten. The skin lesions were highly variable in appearance and included maculopapular lesions, nodules, plaques, and ulcers, which ranged from erythematous to violaceous in color. A history of waxing and waning skin disease and response to topical steroids were not infrequent. In nine patients, skin lesions had been present for several months to three years prior to the diagnosis of lymphoma. Five of these nine patients had previous skin biopsies in the same region that were interpreted as benign or atypical lymphocytic infiltration.

Primary therapy is shown in Table 2. Two patients were never treated. One died of gastrointestinal hemorrhage shortly after diagnostic laparotomy. Another had a spontaneous regression of diffuse adenopathy and arthralgias prior to the planned institution of therapy. Among the 27 patients receiving primary chemotherapy, 21 received Adriamycin in combination with Cytoxan, vincristine, and prednisone with or without methotrexate and bleomycin. The others were treated with Cytoxan, vincristine, and prednisone alone (two patients) or with procarbazine (four patients). Eleven patients were treated with irradiation alone, including two patients with stage IV cutaneous lymphoma; three patients received combined chemotherapy and radiotherapy.

The majority of patients had a morphologic diagnosis of high-grade, diffuse large cell, immunoblastic (14 patients), which could be subcategorized into polymorphic (3 cases), plasmacytoid (3 cases), clear cell (6 cases), and epithelioid (2 cases). Twelve cases were intermediate-grade, diffuse large cell, and eight were diffuse mixed small and large cell. Three patients in the diffuse mixed category were subcategorized as AILD-like. Two of these patients, both males, presented with a clinical syndrome consistent with AILD, including rash, diffuse adenopathy, and systemic symptoms; hepatosplenomegaly was objectively documented in one. Neither had Coomb's-positive anemia or hypergammaglobulinemia. Overall, there was no correlation between morphology and stage, site, or previous history of dermatologic or immunologic disease.

Phenotypic features. Twenty-five cases (61%) expressed a helper cell phenotype (Leu 2^-3^+), and four expressed a cytotoxic-suppressor phenotype (Leu 2^+3^-). Twelve lymphomas were of undefined phenotype, with eight Leu 2^-Leu 3^- and four Leu 2^+Leu 3^+. The cases lacking either helper or cytotoxic/suppressor phenotype expressed two or more T cell antigens, including Leu 1 (2 cases), Leu 4 (6 cases), Leu 5 (5 cases), Leu 9 (6 cases), and did not express B lineage markers. Of the 25 helper cell phenotypes, there was a loss of one or more pan-T cell differentiation antigens in 19. Overall, the Ia antigens were expressed in 30 cases. Immunologic phenotype did not correlate with stage, the presence or absence of cutaneous or other sites of involvement, previous history of an immunologic or dermatologic disorder, or other clinical features. Neither was there any correlation between histologic category and immunophenotype. The spleen, the site of another group of described T cell-related malignancy, T γ lymphoma, was not available for study in the Leu 2^+3^- cases.[29]

Survival data. With a median follow-up of 16 months (range 1 month to 8 years), 18 patients have died; 14 are alive without disease, and 9 are living with disease. The projected actuarial survival for the entire group is 58% at two years (Fig 1). Table 3 describes the 2-year actuarial survival for selected patient groups. Patients with limited-stage disease have an 83% two-year survival, significantly better ($P < .01$) than those with stage III or IV disease. Three limited-stage patients were salvaged with chemotherapy after failing irradiation and/or surgery. The presence or absence of cutaneous disease did not affect survival at two years. A previous history of a dermatologic or immunologic disorder conferred a poor prognosis; however, all of these patients had advanced disease at diagnosis, 64% with stage IV. Neither age nor sex was prognostically significant. Survival comparisons among patients treated with primary irradiation only *v* chemotherapy (with or without Adriamycin) yielded no significant differences. Two-year survival was similar for patients with helper cell phenotype and those with an undefined phenotype. However, due to an increased number of early deaths among patients with lymphomas of undefined phenotype, the Gehan test achieved statistical significance ($P = .03$). Three of four patients with cytotoxic/suppressor phenotypes were dead at 2 years. Loss or maintenance of pan-T cell antigen expression or expression of Ia did not influence survival. Evaluation of the morphological subtypes revealed no significant survival differences, although at the time of this writing, only one of seven patients with monomorphic T cell lymphoma was alive without disease as compared with 13 of the remaining 34 patients.

Table 3. Two-Year Actuarial Survival in TCL Subsets

Characteristic	No. of Patients	Survival (%)
Stage I and II	13	83
Stage III	12	51
Stage IV	16	25
Cutaneous lymphoma	20	51
No cutaneous lymphoma	21	56
History of dermatologic/immunologic disorder	11	27
No history of dermatologic/immunologic disorder	30	73
Helper phenotype	25	61
Cytotoxic/suppressor phenotype	4	—*
Undefined phenotype	12	59
Pan-T antigens expressed	14	55
Loss of pan-T expression	27	55

*Described in text.

DISCUSSION

In this study, we have defined a group of T cell lymphomas on the basis of morphological and immunologic criteria. MF and lymphoblastic lymphoma were excluded on morphologic grounds. Immunologically, all cases expressed at least two T cell antigens with an absence of B cell markers. In contrast to cell-suspension techniques, our method has the advantage of allowing direct comparison of several antibodies as well as identification of cytologic and architectural detail.

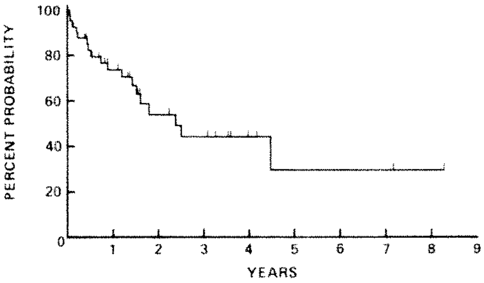

Fig 1. Actuarial survival in 41 patients with T cell lymphoma. Median survival is 29 months with 58% survival at 2 years. Tic marks represent individual patients at risk.

As a group, our 41 TCL patients demonstrate marked clinical, immunologic, and morphological diversity. Several authors have described clinicopathologic features of usually small numbers of patients with TCL as defined by morphological or erythrocyte rosetting techniques. The recent reports of Brisbane and co-workers and Greer and colleagues included multiple histologic subtypes, as in our series.[17,18] Median survivals in these series were 11 months with 70% to 79% of patients in advanced stage. Half or more of the patients in each series had systemic symptoms. In the series of Levine and co-workers, which was restricted to the immunoblastic sarcoma (T-IBS) histologic subtype as defined in the Lukes-Collins system, the median survival of 19 patients was 20 months; 89% of those patients had advanced disease.[13] Grogan and colleagues have recently described nine cases of TCL who were assessed for E rosettes and expression of T cell antigens defined by monoclonal antibodies.[19] Median survival was only 9 months in this elderly group of patients (median age 69 years). These authors have concluded that TCLs are an aggressive subset of the non-Hodgkin's lymphomas, perhaps requiring alternative therapeutic strategies. A broad range of histologic subtypes, often indistinguishable from diffuse non-Hodgkin's lymphomas with B cell or null phenotypes, has been described among the TCLs.[22] It is reasonable then to question whether the T-immunologic features of the intermediate-grade and high-grade lymphomas represent a clinically distinct and significant subset.

Half of our cases had cutaneous lymphoma at diagnosis, in contrast to the series of Greer and colleagues, in which skin involvement was infrequent. However, 6 of 9 patients in Grogan's series had cutaneous involvement. This may be related in part to patient selection as the incidence of dermal involvement could be inflated by the accessibility of cutaneous lymphoma for biopsy. Cutaneous involvement is, of course, a prominent component of other T cell malignancies.

Although the subset of patients with cutaneous lymphoma did not demonstrate unique histologic features or survival data in our series, skin involvement was a prominent aspect of the disease and the exclusive site of lymphoma in 50% of patients. Dermal involvement is usually considered a relatively favorable site of extranodal large cell lymphoma.[30]

A previous history of dermatologic or immunologic disease was noted in 27% of our patients. A history of autoimmune or lymphoproliferative disease has previously been reported in 16% to 36% of patients with TCL, but a history of autoimmune disease is also well described in other non-Hodgkin's lymphomas, particularly the B immunoblastic sarcoma group.[13] Thus, it can be postulated that chronic antigenic stimulation may be related to malignant lymphoma of either B or T cell phenotype.

Three patients in this series had clinical syndromes consistent with AILD. One who later developed clear cell T-IBS had Coomb's-positive anemia and polyclonal gammopathy in addition to adenopathy. Another patient presenting with systemic symptoms, diffuse adenopathy (which was diagnostic of non-Hodgkin's lymphoma and expressed T cell markers) and prominent arthralgias had a spontaneous regression of all disease with a follow-up of 6 months, as has been occasionally described in AILD.[31]

Although the clinical features of TCL patients are indeed heterogeneous, manifestations unique to T cell lymphoma may exist. Myeloid hyperplasia and/or peripheral eosinophilia were seen in three of our patients. Peripheral eosinophilia was noted in 29% of TCL patients reported by Greer and co-workers and is well described in Japanese adult T cell leukemia. Griffin and colleagues have described myeloid colony-stimulating activity in a group of T cell lines, some with preferred stimulation of eosinophilic colonies.[32]

A distinct subset of TCLs associated with the human T cell leukemia/lymphoma virus (HTLV) has been recognized.[33-35] Typical features of this clinicopathologic entity include diffuse adenopathy, hypercalcemia, leukemia at diagnosis or during the clinical course, skin and bone marrow involvement, lytic bone lesions, and eventual involvement of the central nervous system (CNS). In this country, HTLV-associated TCLs have been described predominantly among young blacks born in the southeastern United States. Ninety-three percent of patients in our series are white. Sera from two of our patients were tested for HTLV-specific antibody by Dr Robert Gallo's laboratory at the National Institutes of Health. Both were negative. Only one of our patients (who was HTLV-antibody negative) was diagnosed with hypercalcemia, and none fit this clinical picture.

Descriptions of the clinical features of TCL must be interpreted cautiously with respect to the criteria for diagnosis, selection factors, and the number of cases studied. In the literature, cases have been described as primarily nodal,[6,13,18,20] primarily extranodal,[7,19] with prominent lung or pleural involvement,[6] with systemic symptoms,[6,13,18] without systemic symptoms,[7] with[7,19] or without cutaneous involvement,[18] and of favorable,[7] unfavorable[13,17-19] and indistinctive[23,36] prognosis. In a previous study of the clinical relevance of immunologic phenotype in diffuse large cell lymphoma, we found that stage, age >65 years, systemic symptoms, and marrow involvement had the greatest impact on survival. To address the influence of T cell phenotype better, we compared survival data in the current series of 41 TCL patients with the previous group of 59 cases studied with the same methodology from 1977 to 1982. The latter cases had diffuse large cell morphology, non-T cell phenotypes, and no history of histologic transformation from a low-grade lymphoma. The survival curves of these two groups shown in Fig 2 do not differ significantly. Among these 41 T cell cases and 59 non-T cell cases, the incidence of the following clinical features was: systemic symptoms (27% *v* 37%), age >65 years (29% *v* 25%), stage IV (37% *v* 39%), and marrow + (7% *v* 12%), respectively. Although treatment varied greatly in both groups of patients, these data suggest that the T cell phenotype may not necessarily confer a worse prognosis in a group of patients studied at a single institution.

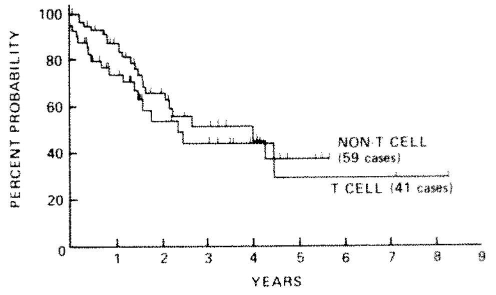

Fig 2. Actuarial survival in 41 patients with lymphomas expressing T cell antigens and 59 patients with lymphomas which do not express T cell antigens. Tic marks represent individual patients at risk.

Neither morphological subtype or antigenic profile correlated with clinical features at presentation or predicted outcome in our study. It is interesting that morphological and immunophenotypic diversity has also been described in HTLV-associated lymphomas, despite similar clinical features.[34] With the exception of the HTLV-related lymphoma/leukemia, TCLs are clinically heterogeneous. Contrary to B cell lymphomas, in our experience, TCLs do not show phenotypes that recapitulate stages of T cell differentiation.[24] In this regard, perhaps the term "peripheral" T cell lymphoma should not be used, because it may be confusing to clinicians treating other T cell malignancies with peripheral blood or nodal involvement. Prospective studies using uniform treatments are necessary to address the clinical significance of the T cell phenotype definitively, independent of established histologic and clinical features.

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REFERENCES

1. Lukes RJ, Collins RD: Immunologic characterization of human malignant lymphoma. Cancer 34:1488, 1974

2. Lukes RJ, Parker JW, Taylor CR, Tindle BH, Cramer AD, Lincoln TL: Immunologic approach to non-Hodgkin's lymphomas and related leukemias: Analysis of the results of multiparameter studies of 425 cases. Semin Hematol 15:322, 1978

3. Lennert K: Malignant Lymphomas Other Than Hodgkin's Disease. Histology. Cytology. Ultrastructure. Immunology. New York, Springer-Verlag, 1978

4. Jaffe ES, Shevach EM, Sussman EH, Frank M, Green I, Berard CW: Membrane receptor sites for the identification of lymphoreticular cells in benign and malignant conditions. Br J Cancer 31:107, 1975 (suppl II)

5. Mann RB, Jaffe ES, Braylan RC, Egglesto JC, Ransom L, Kaiser H, Berard CW : Immunologic and morphologic studies of T cell lymphoma. Am J Med 58:307, 1975

6. Waldron JA, Leech JH, Glick AD, Flexner JM, Collins RD: Malignant lymphoma of peripheral T-lymphocyte origin. Cancer 40:1604, 1977

7. Pinkus GS, Said JW, Hargreaves H: Malignant lymphoma, T cell type: A distinct morphologic variant with large multilobated nuclei, with a report of four cases. Am J Clin Pathol 72:540, 1979

8. The Non-Hodgkin's Lymphoma Pathologic Classification Project: National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: Summary and description of a working formulation for clinical usage. Cancer 49:2112, 1982

9. Palutke M, Tabaczka P, Weise RW, Axelrod A, Palacos C, Margolis H, Khilanani P, Ratanathanathorn V, Piligian J, Pollaud R, Husam M: T-cell lymphomas of large cell type: A variety of malignant lymphomas: "histiocytic" and mixed-lymphocytic-"histiocytic." Cancer 46:87, 1980

10. Borowitz MJ, Brynes RK, Cousar JB, Whitcomb CL, Cuissman JD, Byrne GE, Collins RD: Phenotypic heterogeneity of peripheral T cell lymphomas: The Southeastern Cancer Study Group experience. Lab Invest 50:5, 1984

11. Watanabe S, Nakajima T, Shimosato Y, Shimoyama M, Minato K: T-cell malignancies: Subclassification and interrelationship. Jpn J Clin Oncol 9:423, 1979 (suppl)

12. Shimoyama M, Mirato K, Saito H, Takenaka T, Watanabe S, Nagatani T, Naruto M: Immunoblastic lymphadenopathy (IBL)-like T-cell lymphoma. Jpn J Clin Oncol 9:347, 1979 (suppl)

13. Levine AM, Taylor CR, Schneider DR, Koehler SC, Forman SJ, Lichtenstein A, Lukes RJ, Feinstein DI: Immunoblastic sarcoma of T-cell vs B-cell origin. I. Clinical features. Blood 58:52, 1981

14. Leong AS-Y, Dale BM, Liew SH, Sage RE, Forbes IJ: Node-based T cell lymphoma: The clinical, immunological and morphological spectrum. Pathology 13:79, 1981

15. Nathwani BN, Winberg CD, Rappaport H, Zelman RJ: Malignant lymphoma, T-immunoblastic: A clinicopathologic and immunologic study of 41 patients. Blood 54:201a, 1979 (suppl 1) (abstr)

16. Jaffe ES, Cossman J, Fisher RI: Immunologic, pathologic and clinical analysis of peripheral T-cell lymphomas. Blood:160a, 1980 (suppl 1) (abstr)

17. Brisbane JU, Berman LD, Neiman RS: Peripheral T-cell lymphoma: A clinicopathologic study of nine cases. Am J Clin Pathol 79:285, 1983

18. Greer JP, York JC, Cousar JB, Mitchell RT, Flexner JM, Collins RD, Stein RS: Peripheral T-cell lymphoma: A clinicopathologic study of 42 cases. J Clin Oncol 7:788, 1984

19. Grogan TM, Fielder K, Rangel C, Jolly CJ, Wirt DP, Hicks MJ, Miller TP, Brooks R, Greenberg B, Jones S: Peripheral T-cell lymphoma: Aggressive disease with heterogeneous immunotypes. Am J Clin Pathol 83:279, 1985

20. Brouet J-C, Rabian C, Gisselbrecht C, Flandrin G: Clinical and immunologic study of non-Hodgkin's T-cell lymphomas (cutaneous and lymphoblastic lymphomas excluded). Br J Haematol 57:315, 1984

21. Knowles DM, Halper JP: Human T-cell malignancies. Am J Pathol 106:187, 1982

22. Jaffe ES, Strauchen JA, Berard CW: Predictability of immunologic phenotype by morphologic criteria in diffuse aggressive non-Hodgkin's lymphomas. Am J Clin Pathol 77:46, 1982

23. Horning SJ, Doggett RS, Warnke RA, Levy R: Clinical relevance of immunologic phenotype in diffuse large cell lymphomas. Blood 63:1209, 1984

24. Weiss LM, Crabtree GS, Rouse RV, Warnke RA: Morphologic and immunologic characterization of 50 peripheral T cell lymphomas. Am J Pathol 118:316, 1985

25. Rouse RV, Warnke RA: Special applications of tissue section immunologic staining in the characterization of monoclonal antibodies and in the study of normal and neoplastic tissues, in Weir DM, Blackwell CC, Herzenberg LA (eds): Handbook of Experimental Immunology, 4th ed. Edinburgh, Blackwell (in press)

26. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457, 1958

27. Gehan EA: A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika 52:203, 1965

28. Carbone PP, Kaplan HS, Musshoff K, Smithers DW, Tubiana M: Report of the committee on Hodgkin's disease staging classification. Cancer Res 31:1860, 1971

29. Kadin ME, Kamoun M, Lamberg J: Erythrophagocytic T γ lymphoma: A clinicopathologic entity resembling malignant histiocytosis. N Engl J Med 304:648, 1981

30. Fisher RI, DeVita Jr VT, Johnson B, Simon R, Young RC: Prognostic factors for advanced diffuse histiocytic lymphoma following treatment with combination chemotherapy. Am J Med 63:177, 1977

31. Frizzera G, Moran EM, Rappaport H: Angio-immunoblastic lymphadenopathy: Diagnosis and clinical course. Am J Med 59:803, 1975

32. Griffin JD, Meuer SC, Schlossman SF, et al: T cell regulation of myelopoiesis: Analysis at a clonal level. J Immunol 133:1863, 1984

33. Gallo RC, Kalyanaraman VS, Sarngadharan MG, Sliski A, Vonderheid EC, Maeda M, Nakao Y, Yamada K, Ito Y, Gutensohn N, Murphy S, Bunn PA, Catovsky D, Greaves MF, Blayney DW, Blattner W, Jarrett WHF, zur Hausen H, Seligmann M, Brouet JC, Haynes BF, Jegasothy BV, Jaffee E, Cossman J, Broder S, Fisher RI, Golde DW, Robert-Guroff M: Association of the human type C retrovirus with a subset of adult T-cell cancers. Cancer Res 43:3892, 1983

34. Jaffe ES, Cossman J, Blattner WA, Robert-Guroff M, Blayney DW, Gallo RC, Bunn PA: The pathologic spectrum of adult T-cell leukemia/lymphoma in the United States. Am J Surg Pathol 8:263, 1984

35. Bunn PA, Schecter GP, Jaffe ES, Blayney D, Young RC, Matthews MJ, Blattner W, Broder S, Robert-Guroff M, Gallo RC: Clinical course of retrovirus-associated adult T-cell lymphoma in the United States. N Engl J Med 309:257, 1983

36. Cossman J, Jaffe ES, Fisher RI: Immunologic phenotypes of diffuse, aggressive non-Hodgkin's lymphomas: Correlation with clinical features. Cancer 54:1310, 1984

Monoclonal Antibodies to Human Vitamin K-Dependent Protein S

By Robert D. Litwiller, Richard J. Jenny, Jerry A. Katzmann, Randall S. Miller, and Kenneth G. Mann

Monoclonal antibodies to human protein S have been prepared using established hybridoma technology. One antibody was isolated that binds protein S only when Ca^{2+} is present; others bind antigen equally well in the presence or absence of EDTA. Other antibodies display a diminished affinity for protein S in the presence of EDTA. Purified immunoglobulins from cell lines displaying Ca^{2+} dependence were immobilized and used to purify protein S from fractions obtained by barium precipitation of citrated plasma, ammonium sulfate fractionation, and chromatography on diethylaminoethanol (DEAE)-Sephadex and dextran sulfate agarose. Essentially homogeneous protein S was isolated from the barium-citrate-adsorbed, 35% ammonium-sulfate-soluble proteins using a totally Ca^{2+}-dependent antibody and EDTA elution. Protein S and several substances of higher mol wt were bound directly from plasma by a partially Ca^{2+}-dependent antibody and were eluted partially with EDTA and NaCl and finally with NaSCN. The largest and most abundant of the high mol wt materials is likely protein S-complement C4b-binding protein complex. The immunoaffinity-isolated protein S was found to be indistinguishable from conventionally isolated protein S in terms of activity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mobility, and by high-performance liquid chromatography (HPLC). These studies establish reagents that can probe the structure of protein S and isolate protein S in its free and complexed forms.

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PROTEIN S[2,3] is a vitamin K-dependent protein which exists in human plasma as a free protein and is associated with complement component C4b-binding protein.[4,5] It shows significant NH_2-terminal sequence homology with the other known vitamin K-dependent proteins[6] and contains a second modified amino acid, β hydroxyaspartic acid.[7] Protein S is considered to function as a cofactor for the inactivation of factors V(Va) and VIII(VIIIa) catalyzed by activated protein C (APC).[8-12] The APC-directed proteolytic inactivation of factor Va is enhanced by protein S, which in itself possesses no proteolytic activity.[8] Kinetic data indicate that APC is maximally stimulated by a molar equivalent of protein S, suggesting that APC and protein S combine with a 1:1 stoichiometry to form the factor Va inactivation complex at the phospholipid surface.[8] This conclusion is supported by the ability of protein S to bind to phospholipid vesicles and its ability to enhance the binding of APC to the vesicles, and by the accumulated evidence which suggests that the substrate for APC is phospholipid-bound factor Va or factor VIIIa.[8,13-17] It has recently been shown that protein S is required for the specific binding and activity of APC on bovine platelets, and that platelet activation leads to the expression of either protein S or a similar component that is able to substitute for exogenously added protein S in the enhancement of Va inactivation by APC.[18]

A substantial amount of evidence has shown that APC functions as an anticoagulant, through its ability to inactivate both factor V(Va) and factor VIII(VIIIa).[13,16-19] Patients with a protein S deficiency have exhibited severe recurrent venous thrombosis.[20-22] Two related individuals with otherwise normal coagulation tests were studied; plasma samples obtained were not effectively anticoagulated by APC, but exogenously added protein S restored a normal anticoagulant response.[21]

Recently, monoclonal antibodies have been used to isolate various proteins,[23-26] and have proved to be valuable tools for studying the mechanisms of the prothrombinase system.[27-31] This article describes the generation of monoclonal antibodies to human protein S and their use in identifying Ca^{2+}-dependent conformational changes of protein S, purification of protein S, and the isolation of high mol wt complex from plasma. To demonstrate that immunoaffinity-isolated protein S that had been eluted with sodium thiocyanate still maintained functional activity, the protein S was compared to conventionally isolated protein S for its ability to enhance the inactivation of factor Va by APC. In an experiment similar to that performed by Walker,[9] it was demonstrated that the specific activity of protein S obtained by conventional methods was indistinguishable from that obtained by immunoaffinity chromatography. In addition, this experiment provided the first direct evidence that human protein S behaves analogously to its bovine counterpart relative to its influence on the inactivation of factor Va by APC.

MATERIALS AND METHODS

Materials. Some of the protein S used in these studies was generously provided by Dr S. P. Bajaj.[32] Other vitamin K-dependent proteins were isolated in our laboratory by conventional methods.[32,33] Radioiodination of proteins used in screening assays was done with Bolton-Hunter reagent[34] or with Iodogen[35] (Pierce Chemical Co). Human protein C was activated with thrombin as described by Kisiel et al.[13] Human factor V was isolated by a modification of the Katzmann procedure.[24] Fresh frozen plasma was thawed, and benzamidine-HCl, heparin, and soybean trypsin inhibitor were added to final concentrations of 10 mmol/L, 2 U per milliliter and 20 μg per milliliter, respectively. The plasma was stirred at 4 °C, and 1.0 mol/L of $BaCl_2$ was added (80 mL per liter). After 15 minutes of stirring, solid polyethylene glycol was added to a final concentration of 4% (wt/vol). Following an additional 30 minutes of stirring, the sample was centrifuged at 6,000 g at 4 °C for 30 minutes. Equal volumes of water and 0.025 mol/L of Tris-HCl, 5.0 mmol/L of $CaCl_2$, 1.0 mmol/L of benzamidine-HCl, pH 7.5 were added to the supernatant. Packed QAE cellulose which had been equilibrated

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with 0.025 mol/L of Tris-HCl, 5.0 mmol/L of $CaCl_2$, and 1.0 mmol/L of benzamidine-HCl, pH 7.5, was added (200 mL per liter of plasma), and the suspension was stirred for 30 minutes at 4 °C. The resin was then transferred to a sintered glass funnel for batch chromatography at room temperature. The resin was washed with 0.02 mol/L of Imidazole, 5.0 mmol/L of $CaCl_2$, pH 6.5, followed by a wash with the same buffer containing 0.1 mol/L of NaCl. Following the second wash, the factor V was eluted with the same buffer containing 0.3 mol/L of NaCl. The eluate containing factor V was applied to a column (2.5 × 10 cm) of immobilized antibody to human factor V. After the sample was applied, the column was washed with 0.02 mol/L of Imidazole, 5.0 mmol/L of $CaCl_2$, and 0.3 mol/L of NaCl, pH 6.5, until the absorbance (280 nm) was <0.010. The factor V was then eluted with the same buffer, but 1.5 mol/L in NaCl. Fractions containing factor V were pooled, chilled to 4 °C and concentrated by precipitation with solid ammonium sulfate (0.48 g per milliliter) at 4 °C. To prepare factor Va, a solution of factor V (0.5 mg per milliliter) in 0.02 mol/L of Tris–HCl and 0.15 mol/L of NaCl, pH 7.4, was treated with thrombin for 15 minutes at 37 °C at a final concentration of 6 NIH U per milliliter. The factor Va was treated with dansylarginine-*N*-(3-ethyl-1,5-pentanediyl)amide (DAPA) at a final concentration of 17 nm. The Va was then diluted with 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; and $CaCl_2$ was added to give a final solution of 0.76 μmol/L of Va, containing 2 mmol/L of $CaCl_2$. The Va was then stored on ice and used within five hours. Human factor X was activated to Xa according to a method described for bovine factor X.[36] Thrombin was prepared as described by Lundblad et al.[37] Phospholipid vesicles (PCPS) of 75% phosphatidylcholine and 25% phosphatidylserine were prepared by the procedure of Barenholz and colleagues[38] as described by Higgins and Mann.[39] DAPA was prepared as described by Nesheim and co-workers.[40] Other reagents were obtained commercially and were of analytical quality.

Protein concentrations. The mol wts and extinction coefficients ($E^{1\%}_{1cm}$, 280 nm) of the respective proteins were taken as follows: factor V[41] 330,000, 9.6; prothrombin[42] 70,000, 13.8; factor Xa[2] 46,000, 11.6; activated protein C[43] 60,000, 14.5; protein S[3] 70,000, 10.0.

Solid-phase radioimmunoassay. The assay used to screen culture supernatants has been described[27] previously. In brief, 0.2 mL of supernatant was added to polystyrene tubes that had been coated with rabbit IgG anti-mouse immunoglobulin. After incubation, the tubes were washed three times with 0.02 mol/L of Tris-HCl, 0.10 mol/L of NaCl, and 0.02% $NaNH_3$, pH 7.3, and ^{125}I-protein was added in the same buffer made 0.1% with BSA; in some instances, the buffer was made 2 mmol/L with $CaCl_2$ or with EDTA. After incubation, the tubes were washed three times with 0.02 mol/L of Tris-HCl, 0.10 mol/L of NaCl, 0.02% NaN_3, and 0.1% BSA, pH 7.3, and were counted in a Beckman gamma counter.

Immunization, cell fusion, and cloning. BALB/c mice were initially immunized by intraperitoneal injection of 40 μg of protein S in complete Freund's adjuvant. Six months later, a second injection of 40 μg of protein S in 0.01 mol/L of sodium phosphate, 0.15 mol/L of NaCl, pH 7.4, was given. Immunized spleen cells were fused with murine NS-1 myeloma cells in the presence of polyethylene glycol and distributed into the wells of our 24-well culture plates.[24] Cells in positive wells selected for further study were subcloned by limiting dilution.[27] Those yielding cloned cells were subsequently injected intraperitoneally into BALB/c mice for the production of antibody-rich ascites fluid.[27]

Polyacrylamide gel electrophoresis. Slab gel electrophoresis was run in the presence of sodium dodecyl sulfate (SDS) by the method of Laemmli.[44] Proteins were visualized with Coomassie brilliant blue R-250 and destained by diffusion in 18% methanol-9% acetic acid. ^{125}I-Labeled proteins were visualized by autoradiography.

HPLC. The HPLC system consisted of two Waters 6000 pumps, a Water U6K injector, a Waters 660 programmer, a Waters 441 detector equipped with a zinc lamp, a 214-nm filter, and a Beckman Ultraport RPSC column. Solvent A was 0.05% trifluoroacetic acid (Pierce Chemical Co) in water processed through a Millipore Norganic cartridge. Solvent B was 0.05% TFA in HPLC grade acetonitrile. Proteins were generally injected in 5 to 50 μL of 0.2 mol/L Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, made up to ~100 μL with HPLC buffer A. Elution was achieved with a 15-minute linear gradient from 30% buffer B to 60% buffer B; flowrate was 1.0 mL per minute. Spectra were recorded on a Bascom-Turner series 8000 recorder and in some instances were electronically reduced to fit the format of the figure.

Amino acid analysis. Alkaline hydrolysis was done by the procedure of Price.[45] Analyses were performed by postcolumn derivitization with *o*-phthalaldehyde using an Interaction AA911 column and elution with a linear gradient from 0.2 N sodium citrate, pH 3.28, to 0.15 N NaCl, 0.05 N NaOH, and 0.15 N boric acid, pH 9.8.

Conventional isolation of protein S from plasma. The vitamin K-dependent proteins in citrated plasma were adsorbed onto barium citrate and fractionated with ammonium sulfate. After dialysis against 0.025 mol/L of citrate, 0.10 mol/L of NaCl, and 1 mmol/L of benzamidine–HCl, pH 6, the sample was applied to a DEAE-Sephadex column equilibrated with dialysis buffer. Proteins were eluted by a linear gradient from 0.1 mol/L of NaCl to 0.6 mol/L of NaCl in equilibration buffer.[46] Fractions were pooled based on the elution profile obtained when A_{280} was plotted against fraction number. Pooled fractions were then made 1 mmol/L with DFP, and 5 mmol/L with EDTA, and dialyzed against 20 mmol/L of 2-(*N*-morpholino)-ethane sulfonic acid-Tris, pH 6, containing 1 mmol/L of benzamidine-HCl. The dialysate was made 2.5 mmol/L with $CaCl_2$ and chromatographed on a dextran sulfate agarose column equilibrated with dialysis buffer made 2.5 mmol/L with $CaCl_2$. Proteins were eluted by a linear gradient to 1 mol/L NaCl in the same buffer,[43] with protein S eluting just ahead of the protein C activity.

Isolation of immunoglobulin fraction of murine ascites fluids. The immunoglobulin fraction of murine ascites fluids were isolated by gel filtration chromatography on Ultrogel AcA34 (LKB Instruments) at 24 °C in 0.01 mol/L of sodium phosphate, 0.15 mol/L of NaCl, 0.02% of NaN_3, pH 7.4. The immunoglobulins were precipitated by addition of ammonium sulfate to 70% saturation, collected by centrifugation, and stored in a 1:1 mixture of glycerol and elution buffer.

Purification of protein S with immobilized antibody. Purified anti-protein S IgG was reacted[47] with CNBr-activated Sepharose 4B at 1 mg IgG per milliliter of resin using 0.2 mol/L citrate, pH 6.5, as coupling buffer.

Eluate from a dextran sulfate agarose column that contained protein S was dialyzed against 0.02 mol/L of Tris–HCl, and 0.15 mol/L NaCl, pH 7.4, and applied to the antibody-Sepharose column that had been equilibrated in the same buffer. If the sample volume was less than the void volume of the column, flow was stopped for one hour after the sample was applied. Otherwise, the flow rate was slowed to about 0.3 mL per minute. The column was washed with sample buffer until a constant A_{280} was achieved (≤0.005) and then eluted with one or more of the following buffers: 0.01 mol/L of EDTA; 0.02 mol/L of Tris-HCl; 0.15 mol/L NaCl, pH 7.4; 0.02 mol/L of Tris-HCl; 1.2 mol/L of NaCl, pH 7.4; 3 mol/L of NaSCN; 0.02 mol/L of Tris-HCl; 0.15 mol/L of NaCl, pH 7.4; or with 0.1 mol/L of glycine and 2 mol/L of NaCl, pH 2.8. After use, resins were stored in 0.02 mol/L of Tris-HCl; 0.15 mol/L of NaCl; and 0.02% NaN_3, pH 7.4.

Isolation of protein S from barium citrate-adsorbed, vitamin K-dependent proteins. Citrated plasma[32] was precipitated with barium chloride; the precipitate was suspended in 35% ammonium sulfate, 0.05 mol/L of benzamidine-HCl and centrifuged. After dialysis against 0.02 mol/L of Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, the supernatant was applied to an anti-protein S-Sepharose column that had been equilibrated with dialysis buffer. Flow rate was maintained at about 0.3 mL per minute, and the column was eluted to a stable baseline with 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, with 10 mmol/L of EDTA in the same buffer.

Isolation of high mol wt material from plasma. Plasma from blood drawn into citrate, benzamidine-HCl, soybean trypsin inhibitor anticoagulant[32] was applied directly to an antibody-Sepharose column that had been equilibrated with 0.02 mol/L Tris-HCl and 0.15 mol/L of NaCl, pH 7.4. Flow rate was maintained at about 0.3 mL per minute, and the column was washed with equilibration buffer until a stable baseline was achieved ($A_{280} \leq 0.010$). The column was then eluted successively with 10 mmol/L of EDTA and 0.5 to 2.0 mol/L of NaCl in three increments and 3 mol/L of NaSCN in the same buffer.

Recovery of dilute protein samples. When required prior to analysis, protein samples were concentrated in Centricon 30 microconcentrators (Amicon). Typically, 60% to 70% of the protein was recovered when 8 to 10 mL of sample was concentrated to ~0.25 mL.

Activated protein C and cofactor protein S assay. A two-stage assay was used to measure the cofactor activities of both conventionally isolated protein S, and immunoaffinity-isolated protein S. The factor Va inactivation mixture (final vol 0.056 mL) contained factor Va (13.6 nmol/L), PCPS (10.4 µmol/L), and $CaCl_2$ (2 mmol/L), in 0.020 mol/L of Tris-HCl, 0.15 mol/L of NaCl, pH 7.4, and was briefly incubated at 22 °C in the presence or absence of protein S (38 nmol/L). The reaction was initiated by the addition of APC (7.4 nmol/L). Aliquots (20 µL) were withdrawn at various times after incubation at 37 °C, to determine the amount of factor Va activity remaining by the assay previously described.[40] The assay mixture contained prothrombin (1.49 µmol/L), factor Xa (3.5 nmol/L), $CaCl_2$ (2 mm), PCPS (23 µmol/L) and DAPA (3 µmol/L). The factor Xa concentration was adjusted to a 26-fold molar excess over the highest potential factor Va concentration to assure that Va was limiting and that the assay was directly proportional to Va concentration.

RESULTS

Fifty of the initial 96 wells were determined to be positive for antibody by the solid phase RIA. Eleven showed a varying dependence on Ca^{2+} for the binding of ^{125}I-protein S. The reactivity patterns of the 12 supernatants selected for further study are shown in Fig 1. Of the 12 selected for subcloning, six showed Ca^{2+} dependence (wells 12, 15, 19, 26, 71, and 76), four showed no Ca^{2+} dependence (wells 14, 27, 50, and 87), and two showed a slight increase in binding of ^{125}I-protein S in the presence of EDTA (wells 22 and 48). Seven of the hybrids did not yield cloned cells (wells 12, 14, 22, 26, 27, 48, and 76). Those that gave clones exhibited reactivity patterns as ascites fluids similar to those found in the initial screen; this is apparent in the titration curves shown in Fig 2.

Three of the ascites fluids were checked for cross-reactivity with human vitamin K-dependent proteins prothrombin, factor IX, factor X, and protein C; again ^{125}I-labeled proteins were used in the solid-phase RIA. Results are summarized in Table 1. Only when protein C was added were the cpm bound significantly above background. By dissolving the residues in

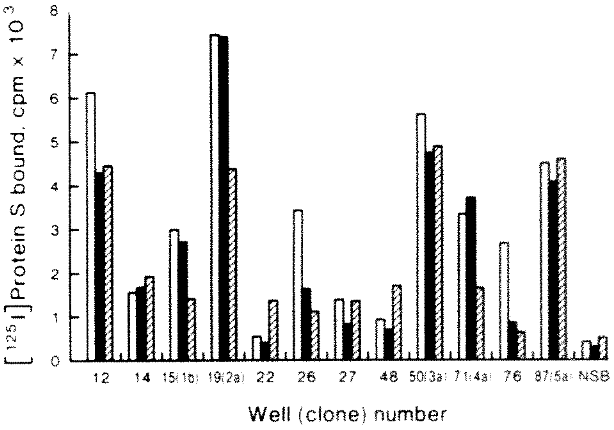

Fig 1. Antibody-positive cultures that were selected for subcloning by limiting dilution: initial screening of supernatants was done by solid-phase radioimmunoassay. The cpm values shown are not corrected for nonspecific binding (NSB), which is given by the last set of bars. Numbers on the abscissa indicate the initial well number followed by the number, in parentheses, assigned to the resulting clone. ^{125}I-Protein S (22,000 cpm) was added in 0.05 mol/L of Tris-HCl, 0.10 mol/L of NaCl, 0.1% bovine serum albumin, and 0.02% NaN_3, pH 7.3 (open bars), in the same buffer made 2 mmol/L with $CaCl_2$ (filled bars) or in the same buffer made 2 mmol/L with EDTA (hatched bars).

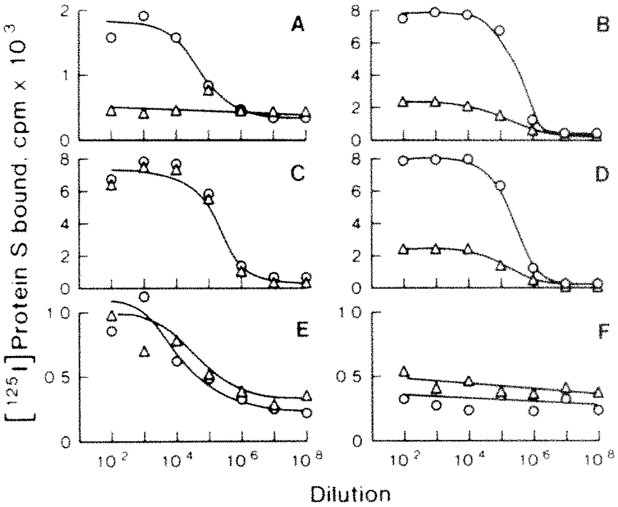

Fig 2. Titration of anti-human protein S ascites fluids with ^{125}I-protein S added in the absence (O—O—O) and in the presence (Δ—Δ—Δ) of EDTA (2 mol/L): dilutions were made in 0.05 mol/L of Tris-HCl, 0.1 mol/L of NaCl, 0.02% NaN_3, pH 7.3, and cpm bound were determined with solid-phase radioimmunoassay. The cpm bound values shown are not corrected for nonspecific binding; lower right panel illustrates nonspecific binding using normal mouse serum. Clones being assayed: (A), 1b; (B), 2a; (C), 3a; (D), 4a; (E), 5a; (F), NMS. These clonal designations are also indicated in parentheses on the abscissa in Fig 1.

Table 1. Reactivity of Anti-Human Protein S With Human Prothrombin, Factor IX, Factor X, and Protein C

$[^{125}I]$-Protein	cpm Bound in Solid-Phase RIA					Total cpm Added
	Buffer only	Normal Mouse Serum*	Ascites Fluid 1b*	Ascites Fluid 2b*	Ascites Fluid 3a*	
Prothrombin	266	150	211	238	222	57,528
Factor IX	305	184	363	429	301	52,340
Factor X	180	165	269	239	232	43,304
Protein C	213	269	456	1,192	763	51,180
Protein S	319	349	1,654	7,481	6,986	49,554

RIA, radioimmunoassay.

*Diluted 1:100 with 0.05 mol/L of Tris-HCl, 0.10 mol/L of NaCl, and 0.02% NaN_3, pH 7.3, and then added to the rabbit anti-mouse Ig RIA tubes.

the tubes in 0.0625 mol/L of Tris-HCl, 0.2% and SDS, pH 6.8, separating the ^{125}I-proteins electrophoretically, and visualizing them with autoradiography, it was determined that the counts being bound were due to contaminating protein S in the protein C preparation (data not shown).

Immunoglobulins were isolated from ascites fluids 1b and 2a by gel filtration, and the isolated immunoglobulins were immobilized on Sepharose 4B. Ascites fluid 1b yielded 3 mg IgG per milliliter of fluid and was coupled at 0.7 mg IgG per milliliter of resin. Ascites fluid 2a yielded 6 mg IgG per milliliter of fluid and was coupled at 0.9 mg IgG per milliliter of resin. Both immobilized antibodies were used to purify protein S, which had been isolated from plasma by barium citrate adsorption of the vitamin K-dependent proteins, a two-step ammonium sulfate fractionation, and chromatography on DEAE-sephadex and dextran sulfate agarose.

Immobilized antibody 2a bound about 250 μg of protein S/mg IgG (255 μg per milliliter of resin). Bound protein S was partially eluted with 0.01 mol/L of EDTA; 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; or with 0.02 mol/L of Tris-HCl, and 1.2 mol/L of NaCl, pH 7.4, and was totally eluted with 3.0 mol/L of NaSCN, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; or with 0.10 mol/L of glycine and 2.0 mol/L of NaCl, pH 2.8. After reequilibration with 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4, columns of immobilized antibody 2a were reused repeatedly.

The HPLC analysis of an experiment in which partially purified protein S (in 13 mL of 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, $A_{280} = 0.298$) was applied to a 9-mL column of immobilized antibody 2a is shown in Figs 3 and 4. The migration of four vitamin K-dependent proteins is shown in the bottom profile (Fig 3); in this system, factor VII coeluted with prothrombin in protein C eluted just after factor IX. The major component in the sample (middle profile, Fig 3) comigrated with protein S; however, the sample was significantly contaminated with materials that comigrated with factor X, prothrombin, and factor IX, and with a material that eluted just ahead of protein S. In the flow-through from the column (top profile, Fig 3), the protein S peak was greatly diminished relative to the other peaks.

The column was eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; and with 3.0 mol/L of NaSCN, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4; HPLC analysis of the eluted material (Fig 4) showed a single peak that comigrated with authentic protein S. Typical γ carboxyglutamic acid (Gla) analyses of the antibody-purified protein S gave 9.5 to 12.5 Gla per molecule, based on a total of 63 glutamyl residues.[3]

Polyacrylamide gel analysis of material eluted from an antibody 2a column with EDTA is shown in Fig 5. On the nonreduced gel, the antibody-purified material (lane 3) comigrated as a single band with antigen protein S (lane 2). On the reduced gel, the antibody-purified material (lane 6) migrated as a doublet; this observation is consistent with the findings of Dahlbäck,[48] who reported reduced bands with apparent mol wts of 85,000 and 75,000. The ratio of the two reduced forms of protein S varied from preparation to preparation and in some preparations, minor reduced bands in the range of 30,000 to 40,000 mol wt were observed.

Immobilized antibody 1b bound about 100 μg of protein S/mg IgG (70 μg per milliliter of resin) and, in contrast with antibody 2a, bound material was totally eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl, and 0.15 mol/L of NaCl, pH 7.4. After reequilibration with 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, immobilized antibody 1b was reused repeatedly.

Dahlbäck and Stenflo[4] reported that the complement C4b binding protein–protein S complex is precipitated by 35%

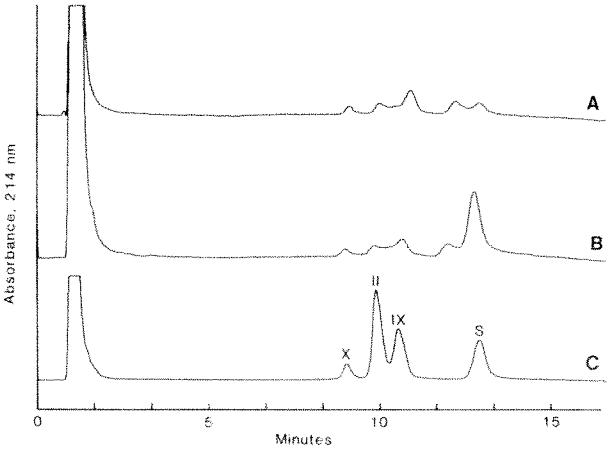

Fig 3. High-performance liquid chromatography analysis of a depletion of human protein S on anti-protein S (2a). Experimental details are given in the text. Standard vitamin K-dependent proteins (C) were isolated conventionally. The sample applied to the antibody column (B) was impure protein S from a conventional isolation as described in the Materials and Methods section. The column flow-through (A) represents material not bound by the anti-protein S.

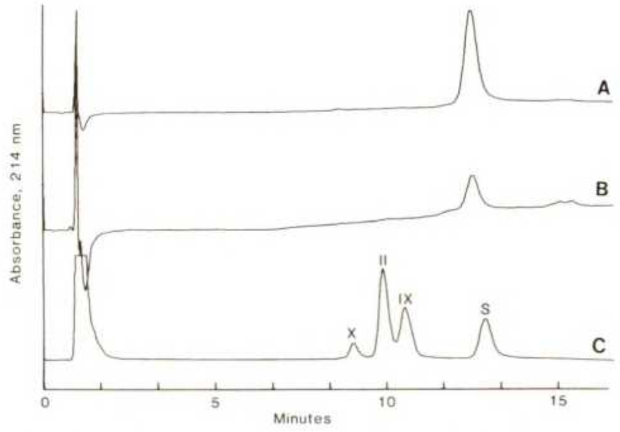

Fig 4. High-performance liquid chromatography analysis of material bound by anti-protein S (2a) and eluted with 0.01 mol/L of EDTA and 3.0 mol/L of NaSCN. Experimental details are given in the text. EDTA-eluted (B) and NaSCN-eluted (A) materials were dialyzed against 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, to remove EDTA and NaSCN. (C), standards.

ammonium sulfate whereas the bulk of the free vitamin K-dependent proteins are not. In light of this observation, the barium citrate-adsorbed material from 22 mL of plasma was suspended in 35% ammonium sulfate containing benzamidine hydrochloride. After 2 hours at 4 °C, the suspension was centrifuged. The supernatant was dialyzed against 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and applied to a 4-mL column of immobilized antibody 1b. Bound material was eluted with 0.01 mol/L of EDTA, 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and the portion which represented ~3 mL of plasma was subjected to slab gel electrophoresis (Fig 6). On both the nonreduced and reduced gels, the antibody-isolated material (lanes 2 and 4) comigrated with the conventionally isolated protein S (lanes 1 and 3) and was essentially homogeneous.

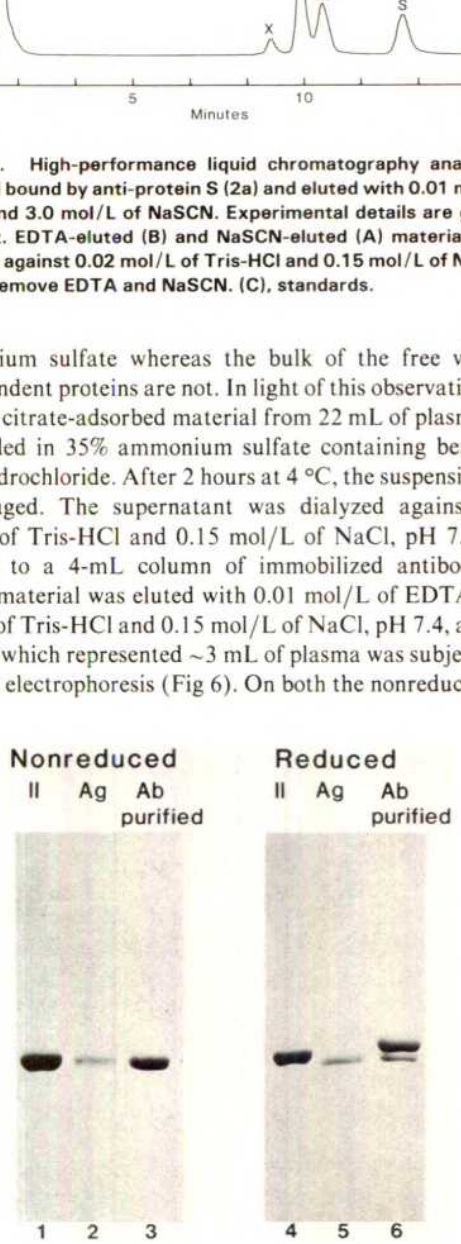

Fig 5. Electrophoretic analysis of material bound by anti-protein S (2a) and eluted with 0.01 mol/L of EDTA. Slab gels of polyacrylamide, 5% to 15% linear gradients, were run in the presence of sodium dodecyl sulfate. Prothrombin was applied to lanes 1 and 4, conventionally isolated protein S used as antigen was applied to lanes 2 and 5, and antibody-purified protein S was applied to lanes 3 and 6.

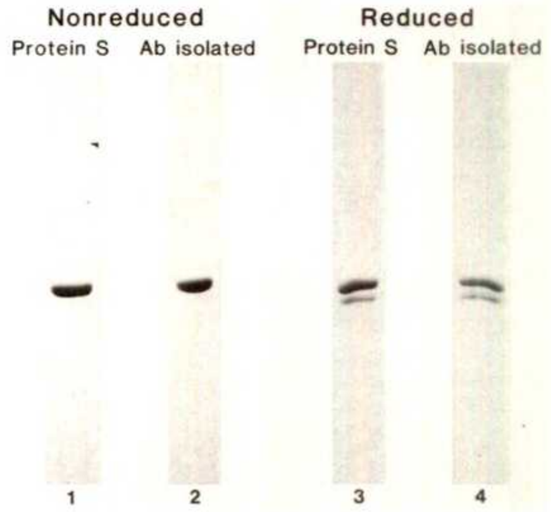

Fig 6. Electrophoretic analysis of material bound by anti-protein S (1b) from the 35% ammonium sulfate supernatant of barium citrate-adsorbed plasma components and eluted with EDTA. Slab gels of polyacrylamide, 5% to 15% linear gradients, were run in the presence of sodium dodecyl sulfate. Conventionally isolated, antibody-purified protein S was applied to lanes 1 and 3; antibody-isolated material was applied to lanes 2 and 4.

Whole citrated plasma was applied to immobilized antibody 2a, and the column was eluted with 0.02 mol/L of Tris-HCl and 0.15 mol/L of NaCl, pH 7.4, and then sequentially with the same buffer made 0.01 mol/L with EDTA, 0.5 and 1.0; 2.0 mol/L with NaCl; and finally 3.0 mol/L with NaSCN. When 11 mL of plasma was applied to a 9-mL column of immobilized antibody 2a, 0.06 A_{280} units was eluted with 0.01 mol/L of EDTA, 0.04 A_{280} units was eluted with 0.5 mol/L of NaCl, and 1.09 A_{280} units was eluted with 3 mol/L of NaSCN. The Gla analysis of the NaSCN-eluted material gave 1.3 Gla per 100 glutamyl residues. Gel electrophoretic analysis of the eluted material is shown in Fig 7. The amount of NaSCN-eluted material loaded on the gel (lanes 1 and 5) represents material from -0.4 mL of plasma; the EDTA-eluted (lanes 3 and 7) and NaCl-eluted (lanes 4 and 8) materials represent material from ~2.5 mL of plasma.

When samples were analyzed without reduction of disulfide bonds, only the NaSCN-eluted material (lane 1) gave a free protein S band on the Coomassie Blue-stained gel. The bulk of the NaSCN-eluted material in nonreduced samples had an apparent mol wt >300,000; EDTA and NaCl-eluted material contained only a trace of material of that size. All immune column eluates, when analyzed with disulfide bonds intact, and two to three components of intermediate mol wt (150,000 to 200,000). The EDTA-eluted material had several components with apparent mol wts that were less than that of protein S.

After reduction of the disulfide bridges, the majority of the NaSCN-eluted material (lane 5) migrated slightly faster than the smaller component protein S (lane 6). This band

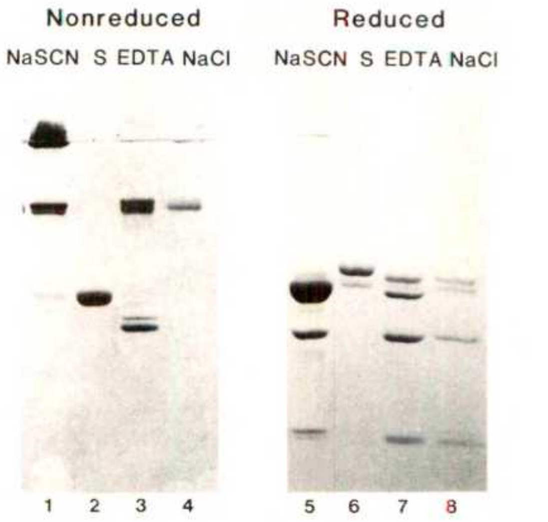

Fig 7. Electrophoretic analysis of material bound from whole citrated plasma by anti-protein S (2a) and eluted with EDTA, NaCl, and NaSCN. Slab gels of polyacrylamide, 5% to 15% linear gradients, were run in the presence of sodium dodecyl sulfate. Protein S, conventionally isolated and antibody-purified, was applied to lanes 2 and 6. Antibody-bound plasma components which were eluted with 0.01 mol/L of EDTA were applied to lanes 3 and 7; those eluted with 0.5 mol/L of NaCl were applied to lanes 4 and 8; and those eluted with 3.0 mol/L of NaSCN were applied to lanes 1 and 5.

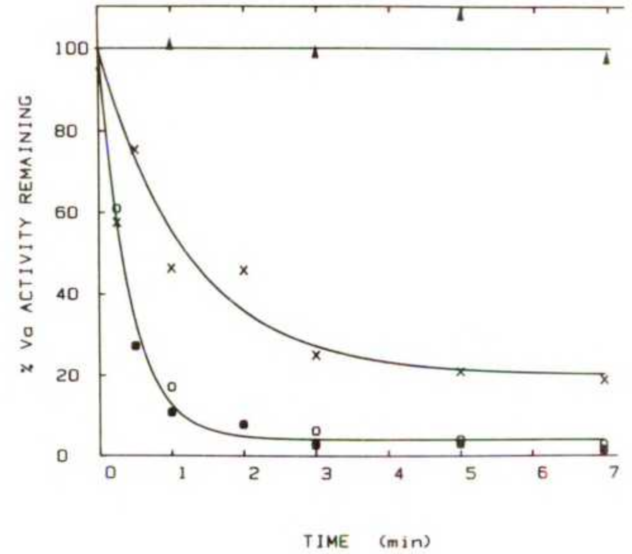

Fig 8. Effect of conventionally isolated, and immunoaffinity-isolated protein S on the inactivation of factor Va by activated protein C. The amount of factor Va activity remaining after incubation in the presence or absence of protein S and with or without activated protein C is plotted *v* incubation time. The inactivation mixtures contained 13.6 nmol/L of factor Va with: no added protein (▲); 7.4 nmol/L of activated protein C (×); 7.4 nmol/L of activated protein C and 39 nmol/L of conventionally isolated protein S (○); and 7.4 nmol/L of activated protein C and 39 nmol/L of immunoaffinity-isolated protein S (●). Protein S alone had no effect on Va activity (data not shown).

was also present in the reduced EDTA-eluted material (lane 7) but was not apparent in the reduced NaCl-eluted material (lane 8). Present in all antibody eluates analyzed with disulfide bonds reduced were components of mol wt 50,000 and 25,000, which probably represent the heavy and light chains of human immunoglobulin that binds nonspecifically to the immobilized mouse immunoglobulin.

The immunoaffinity-isolated protein S proved to have the same relative activity as conventionally isolated protein S in an assay system designed to measure the cofactor activity of protein S as shown in Fig 8. Human protein S obtained by either isolation method enhanced the inactivation of factor Va by APC to an extent consistent with that for the analogous bovine system.[9]

DISCUSSION

Using hybridoma technology, monoclonal antibodies have been raised against human protein S. The affinity of the different antibodies for antigen varies relative to the availability of Ca^{2+}. Antibodies 3a and 5a bind antigen equally well in the presence or absence of EDTA. Antibodies 2a and 4a bind antigen significantly less in the presence of EDTA, and antibody 1b does not bind at all in the presence of EDTA. It is likely that protein S, like other vitamin K-dependent proteins,[49] undergoes a conformational alteration that is stabilized by Ca^{2+}. The partial Ca^{2+} dependence of antibodies 2a and 4a probably occurs because they react with epitopes that are not Ca^{2+}-dependent per se, but that are stabilized by Ca^{2+}, whereas the Ca^{2+}-dependent antibody 1b probably interacts with a truly Ca^{2+}-dependent epitope. Let us suppose that protein S undergoes the transition:

$$S \rightleftharpoons S^*$$

$$K_1 = \frac{[S^*]}{[S]}.$$

If Ca^{2+} interacts preferentially with S^*, then:

$$S^* + nCa^{2+} \rightleftharpoons S^*(Ca^{2+})n$$

$$K_2 = \frac{[S^*\ (Ca^{2+})n]}{[S^*]\ [Ca^{2+}]n}.$$

If 2a and 4a react preferentially with S^*, they will show a mixed Ca^{2+}-dependence since they will react with both S^* and with $S^*(Ca^{2+})n$. The Ca^{2+}-specific antibody 1b would only react with $S^*(Ca^{2+})n$ and its concentration would be expressed by:

$$[S^*(Ca^{2+})n] = K_1K_2[S]\ [Ca^{2+}]n.$$

Antibodies that are Ca^{2+}-dependent are particularly useful for isolating protein S since recovery of antibody-bound protein can be achieved, at least in part, by elution with EDTA. Inhomogeneous, conventionally isolated protein S was purified on antiprotein S-sepharose (Figs 3 through 5). Essentially homogeneous protein S was isolated with immobilized antibody from the 35% ammonium sulfate supernatant of barium citrate-adsorbed, vitamin K-dependent proteins (Fig 6).

Protein S-complement C4b-binding protein complex has been isolated from plasma by adsorption of the vitamin K-dependent proteins on barium citrate followed by chromatography on DEAE-Sephacel[48] and heparin-Sepharose.[5] It is likely that the material of highest mol wt that was isolated directly from plasma with immobilized antibody (Fig 7, lanes 1 and 5) is this complex. The electrophoretic mobility found is consistent with the nonreduced mol wt of 570,000 and a reduced mol wt of 70,000 of complement C4b-binding protein.

The identities of the materials of intermediate nonreduced mol wt (Fig 7, lanes 1, 3, and 4) and the materials that are eluted with EDTA and have an apparent mol wt that is less than that of protein S (Fig 7, lane 3) are not known; some are potentially heterophile human immunoglobins which bind to the mouse immunoglobin on the affinity resin, however.

The use of denaturants such as sodium thiocyanate for the elution of antigen from immunoaffinity chromatography columns has proved to be effective for isolating biologically active protein.[50] Homogenous protein S that had been eluted from immobilized antibody 2a with 3 mol/L of sodium thiocyanate was dialyzed to remove the denaturant and was then assayed for cofactor activity. The fact that this protein S was shown to be biologically active clearly demonstrates the usefulness of this procedure for isolating protein S. In addition, the results show directly for the first time that human protein S provides the same cofactor activity toward the factor Va inactivation by APC as does its bovine counterpart.[9]

The reagents established in this article should be useful in the further investigation of protein S binding substances and in the continuing study of protein S and its role in the coagulation and complement cascades.

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REFERENCES

1. Litwiller RD, Jenny RJ, Mann KG: Monoclonal antibodies to human protein S. Blood 64:264a, 1984 (abstr)

2. DiScipio RG, Hermodson MA, Yates SG, Davie EW: A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor) and protein S. Biochemistry 16:698, 1977

3. DiScipio RG, Davie EW: Characterization of protein S, a γ-carboxyglutamic acid containing protein from bovine and human plasma. Biochemistry 18:899, 1979

4. Dahlbäck B, Stenflo J: High molecular weight complex in human plasma between vitamin K-dependent protein S and complement component C4b-binding protein. Proc Natl Acad Sci 78:2512, 1981

5. Dahlbäck B: Purification of human C_{4b}-binding protein and formation of its complex with vitamin K-dependent protein S. Biochem J 209:847, 1983

6. Broze GJ Jr, Miletich JP: Human protein Z. J Clin Invest 73:933, 1984

7. Fernlund P, Stenflo J: β-Hydroxyaspartic acid in vitamin K-dependent proteins. J Biol Chem 258:12509, 1983

8. Walker FJ: Regulation of activated protein C by protein S. J Biol Chem 256:11128, 1981

9. Walker FJ: Regulation of activated protein C by a new protein. J Biol Chem 255:5521, 1980

10. Marlar RA, Kleiss AJ, Griffin JH: Mechanism of action of human activated protein C, a thrombin-dependent anticoagulant enzyme. Blood 59:1067, 1982

11. Fulcher CA, Gardiner JE, Griffin JH, Zimmerman TS: Proteolytic inactivation of human factor VIII procoagulant protein by activated human protein C and its analogy with factor V. Blood 63:486, 1984

12. Nesheim MN, Canfield WM, Kisiel W, Mann KG: Studies of the capacity of factor Xa to protect factor Va from inactivation by activated protein C. J Biol Chem 257:1443, 1982

13. Kisiel W, Canfield WM, Ericsson LH, Davie, EW: Anticoagulant properties of bovine plasma protein C following activation by thrombin. Biochemistry 16:5824, 1977

14. Canfield W, Nesheim ME, Kisiel W, Mann KG: Proteolytic inactivation of bovine factor Va by bovine activated protein C. Circulation 58(II):II-210, 1978 (abstr)

15. Walker FJ, Sexton PW and Esmon CT: The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. Biochim Biophys Acta 571:333, 1979

16. Suzuki K, Stenflo J, Dahlbäck B, Theodorsson B: Inactivation of human coagulation factor V by activated protein C. J Biol Chem 258:1914, 1983

17. Walker FJ: Regulation of vitamin K-dependent protein S. J Biol Chem 259:10335, 1984

18. Harris KW, Esmon CT: Protein S is required for bovine platelets to support activated protein C binding and activity. J Biol Chem 260:2007, 1985

19. Vehar GA, Davie EW: Preparation and properties of bovine factor VIII (antihemophilic factor). Biochemistry 19:401, 1980

20. Comp PC, Esmon CT: Recurrent venous thromboembolism in patients with a partial deficiency of protein S. N Engl J Med 311:1525, 1984

21. Comp PC, Nixon RR, Cooper MR, Esmon CT: Familial protein S deficiency is associated with recurrent thrombosis. J Clin Invest 74:2082, 1984

22. Schwarz HP, Fischer M, Hopmeier P, Batard MA, Griffen JH: Plasma protein S deficiency in familial thrombotic disease. Blood 64:1297, 1984

23. Staehelin T, Hobbs DS, Kung H, Lai CY, Pestka S: Purification and characterization of recombinant human leukocyte interferon (IFLrA) with monoclonal antibodies. J Biol Chem 256:9750, 1981

24. Katzmann JA, Nesheim ME, Hibbard LS, Mann KG: Isolation of functional human coagulation factor V by using a hybridoma antibody. Proc Natl Acad Sci 78:162, 1981

25. Fass DN, Knutson GJ, Katzmann JA: Monoclonal antibodies to porcine factor VIII coagulant and their use in the isolation of active coagulant protein. Blood 59:594, 1982

26. Stenner DD, Romberg RW, Tracy RP, Katzmann JA, Riggs BL, Mann KG: Monoclonal antibodies to native noncollagenous bone-specific proteins. Proc Natl Acad Sci 81:2868, 1984

27. Foster WB, Katzmann JA, Miller RS, Nesheim ME, Mann KG: Monoclonal antibodies selective for the functional states of bovine factor V and factor Va. Thromb Res 28:649, 1982

28. Tucker MM, Foster WB, Katzmann JA, Mann KG: A monoclonal antibody which inhibits the factor Va:factor Xa interaction. J Biol Chem 258:1210, 1983

29. Foster WB, Tucker MM, Katzmann JA, Mann KG: Monoclonal antibodies selective for activated factor V. J Biol Chem 258:5608, 1983

30. Foster WB, Tucker MM, Katzmann JA, Miller RS, Nesheim ME, Mann KG: Monoclonal antibodies to human coagulation factor V and factor Va. Blood 61:1060, 1983

31. Foster WB, Nesheim ME, Mann KG: The factor Xa-catalyzed activation of factor V. J Biol Chem 258:13970, 1983

32. Bajaj SP, Rapaport SI, Maki SL, Brown SF: A procedure for isolation of human protein C and protein S as by-products of the purification of factors VII, IX, X and prothrombin. Prep Biochem 13:191, 1983

33. Bajaj SP, Rapaport SI, Prodanoc: A simplified procedure for purification of human prothrombin, factor IX and factor X. Prep Biochem 11:397, 1981

34. Bolton AE, Hunter WM: The labeling of proteins to high specific radioactivity by conjugation to a ^{125}[I]-containing acylating agent. Biochem J 133:529, 1973

35. Fraker PS, Speck JC: Protein and cell membrane iodinations with a sparingly soluble chloramide 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochem Biophys Res Commun 80:849, 1978

36. Jesty J, Nemerson Y: The activation of bovine coagulation factor X. Methods Enzymol 45B:95, 1976

37. Lundblad RL, Kingdon HS, Mann KG: Thrombin. Methods Enzymol 45B:156, 1976

38. Barenholz Y, Gibbs D, Litman BJ, Goll J, Thompson TE, Carlson FD: A simple method for the preparation of homogeneous phospholipid vesicles. Biochemistry 16:2806, 1977

39. Higgins DL, Mann KG: The interaction of bovine factor V and factor V-derived peptides with phospholipid vesicles. J Biol Chem 258:6503, 1983

40. Nesheim ME, Prendergast FG, Mann KG: Interaction of a fluorescent active-site-directed inhibitor of thrombin: Dansylarginine *N*-(3-ethyl-1,5-pentanediyl) amide. Biochemistry 18:996, 1979

41. Nesheim ME, Myrmel KH, Hibbard L, Mann KG: Isolation and characterization of single chain bovine factor V. J Biol Chem 254:508, 1979

42. Kisiel W, Hanahan DJ: Purification and characterization of human factor II. Biochim Biophys Acta 304:103, 1973

43. Kisiel W: Human plasma protein C. J Clin Invest 64:761, 1979

44. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) 227:680, 1970

45. Price PA: Analysis of γ-carboxyglutamic acid, in Hirs CHW, Timasheff SN (eds): Methods in Enzymology, Vol 91. New York, Academic Press, 1983, p 13

46. Bajaj SP, Rapaport SI, Brown SF: Isolation and characterization of human factor VII. J Biol Chem 256:253, 1981

47. March SC, Parikh I, Cuatrecasas P: Simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal Biochem 60:149, 1974

48. Dahlbäck B: Purification of human vitamin K-dependent protein S and its limited proteolysis by thrombin. Biochem J 209:837, 1983

49. Prendergast FG, Mann KG: Differentiation of metal ion-induced transitions of prothrombin fragment 1. J Biol Chem 252:840, 1977

50. Church WR, Mann KG: A simple purification of human factor X using a high affinity monoclonal antibody immunoadsorbant. Thromb Res 38:417, 1985

Clinical Evaluation of Platelet Concentrates Stored for One to Five Days

By Charles A. Schiffer, Edward J. Lee, Paul M. Ness, and James Reilly

There are no large-scale data available describing the increments obtained with platelet concentrates stored for varying durations. Platelet concentrates prepared by standard techniques were stored at 22 °C with horizontal agitation in PL-732 bags and administered to clinically stable, nonalloimmunized recipients known to respond well to random donor platelet transfusions. The platelet concentrates were stored in a mean volume of 65.0 mL (range 54–80 mL) with an average yield of $.72 \times 10^{11}$ platelets per unit of platelet concentrates (N = 100 consecutive units). There was no significant deterioration of pH during storage. Mean corrected count increments ranged between 16,600 (N = 146 transfusions) after 1 day of storage to 13,300 (N = 34 transfusions) after 5 days of storage. Although these differences were statistically significant ($P < .003$, analysis of variance), the overall deterioration in increments was quite modest. Platelet concentrates can be stored under standard conditions for 1 to 5 days with acceptable clinical results.

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UNTIL A FEW YEARS AGO, platelet concentrates could be stored at 22 °C to 24 °C for a maximum of three days. The development of new plastics and thinner plastic bags which allowed increased oxygen transport with preservation of aerobic metabolism has permitted further extension of storage initially to five days,[1-3] and more recently to seven days.[4,5] The licensing of these containers was based on a relatively small number of studies which, in general, compared platelet storage for extended periods with results derived from platelets prepared in the same fashion and stored at room temperature for 72 hours in the "standard" polyvinyl chloride plastic bags. Implicit in this decision was the assumption that 72-hour storage was "state of the art" and platelets thus stored were equivalent to platelets which were either fresh or stored for <24 hours. In addition, only a relatively small number of observations were provided, some of which consisted of transfusions of autologous labeled platelets to normal volunteers.

Notably lacking in the medical literature are the results in the clinical setting of platelets which have been transfused after any duration of storage.[6] Thus, although a number of research laboratories have published results in small numbers of patients using platelets stored for 72 hours,[7,8] there are no larger descriptions of clinical results in a more routine, less research-oriented setting. Data are also lacking for more extended storage durations. Although it is difficult to document, one frequently has the impression that clinicians have been distrustful about the effectiveness of stored platelets and frequently either demanded fresh platelets, single-donor platelets, or increased the number of units ordered when stored platelets were administered.[6]

The present study was undertaken shortly after "five-day" platelet bags were licensed in an attempt to provide further information about posttransfusion results after varying durations of storage.

MATERIALS AND METHODS

Platelet concentrates were collected and stored in CPDA-1 plasma at the Chesapeake Regional Red Cross using standard techniques. All platelets were collected in storage bags which were manufactured by Fenwal Laboratories, Morton Grove, Ill (Fenwal PL-732 bags), which at that time were licensed for five-day storage. Whole blood was centrifuged at 2,170 g × three minutes with centrifugation of the platelet-rich plasma (PRP) at 3,450 g × six minutes. Platelets were delivered to the University of Maryland Cancer Center 12 to 24 hours after preparation. At both the Red Cross and the UMCC, the platelets were stored in a Forma Platelet Incubator at 22 °C with gentle horizontal agitation (70 rpm/min). Prior to transfusion, multiple ABO identical units were pooled (between 4 to 10 U depending on the recipient's size) and administered through standard blood filters within one hour of pooling. In this study, only platelets that had been stored for the same duration of time were pooled in a single bag. Specimens for platelet count were obtained on the pooled bag prior to transfusion.

The UMCC has a policy of prophylactic platelet transfusion with patients transfused at platelet counts of 10,000 to 20,000/μL when further decrements in platelet count due to chemotherapy or underlying disease are expected to occur.[9] Evaluated transfusions were administered to recipients who were clinically stable without evidence of severe infection, hepatosplenomegally, disseminated intravascular coagulation or severe bleeding, and who had a temperature <101 °C as described previously.[4] Posttransfusion specimens were obtained one hour after transfusion and usually on the next day, 18 to 24 hours after transfusion. Patients were known to be nonalloimmunized as documented serologically by negative lymphocytotoxic antibody titers[10] and had known adequate responses to random-donor platelet transfusions administered within 1 week of the study transfusions (one hour posttransfusion corrected count increments (CCI) $\geq 7{,}500$). Increments were expressed as CCI where: CCI = Absolute increment × body surface area (m^2)/Number of platelets transfused × 10^{11}. Thus, if a 2 m^2-individual received 4×10^{11} platelets and had an absolute count increment of 40,000, the CCI = 40,000 × 2 = 20,000.

In vitro studies. Specimens were obtained aseptically from units stored 1, 2, 3, 4, or 5 days, and platelet count and pH were determined. No air was allowed in the collection syringes, and the syringes were sealed immediately, thereby minimizing any pH effect that might be incurred by loss of CO_2 from the plasma to surrounding air.[11] One hundred units of platelet concentrate (PC) were analyzed for each of the storage intervals.

RESULTS

The platelet concentrates were stored in a mean volume of 65.0 mL (range 54 to 80 mL) with an average yield of $.72 \times 10^{11}$ platelets per unit of platelet concentrate (data

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Table 1. Platelet Concentrate pH During Storage

	Duration of Storage (Days)				
	1	2	3	4	5
Mean pH (Range)	7.36 (7.14–7.55)	7.35 (7.08–7.66)	7.35 (7.07–7.66)	7.26 (6.9–7.57)	7.29 (6.9–7.6)
Units (%) with platelet counts $>1.5 \times 10^6/\mu L$	19	25	24	21	17
Units (%) with pH > 7.40	20	30	25	14	16

N = 100 for each of the five days of storage.

from 100 consecutive units). As can be seen in Table 1, there was no significant deterioration of pH during storage. Of note is that between 17% to 25% of the units tested at different storage intervals had relatively high platelet concentrations of $>1.5 \times 10^6/\mu L$. The lowest pH noted in the entire study was 6.9, suggesting that the improved oxygen transport prevented the development of significant anaerobic metabolism even at higher platelet concentrations. In contrast, between 14% to 30% of units had elevated pH (>7.4) following storage (Table 1). As would be expected, this was most common in units with lower platelet concentrations ($<1 \times 10^6/\mu L$).

The corrected count increments are shown in Table 2. Results are presented both for the total number of evaluable transfusions as well as for the transfusions administered to nonsplenectomized patients in each group. These latter data are provided because of a fortuitous disproportion of splenectomized recipients in the one- to three-day storage groups. There was a statistically significant effect of storage on CCI in both groups which began after three days of storage ($P < 0.003$, analysis of variance). The overall deterioration was quite modest, however. The frequency of transfusions with CCI <7,500 are presented for the nonsplenectomized patients. Although there was an increased frequency of such transfusion "failures" after three to five days of storage, only 10% of transfusions stored for these durations had CCI of <6,500.

The 18- to 24-hour posttransfusion data are shown in Table 3. Not all patients had counts performed the next day. Most often, these were patients who had been transfused as outpatients and in whom, because one-hour increments had been acceptable, it had not been clinically necessary or appropriate to obtain counts the next day. Therefore, the 18- to 24-hour data might have a slight bias against patients with the highest one-hour posttransfusion increments. In addition, there was a greater variability in the interval posttransfusion than in the one-hour posttransfusion data. Nonetheless, the results were similar with a statistically significant effect ($P < 0.02$, analysis of variance) for the total group. The difference was not statistically significant ($P = 0.19$, analysis of variance) in the group of nonsplenectomized patients.

The ratios of the 18- to 24-hour increments to the corresponding one-hour increments are shown in Table 4. There was no significant difference when either all cases or nonsplenectomized patients were considered ($P = 0.39$ and 0.19, respectively). On average, the one-day increments were approximately two-thirds of the immediate posttransfusion increments.

DISCUSSION

These data demonstrate that acceptable posttransfusion increments can be obtained after five days of storage in Fenwal PL-732 bags with evidence of minimal deterioration over time. It is to be emphasized that the present study was performed using standard collection and storage techniques, and therefore should be similar to other transfusion settings. In a previous study performed 2 to 3 years earlier in a similar patient population at our institution,[4] PC stored for three days in the then-standard plastic bags (CL-3861, Cutter Labs, Berkeley, Calif) had a mean one-hour posttransfusion

Table 2. One-Hour Corrected Count Increments

	Duration of Storage (Days)				
	1	2	3	4	5
Total patients					
1-h posttransfusion CCI (range)	16.6 ± 7.4* (4.1–42.5)	17.9 ± 7.7 (2.7–40.5)	15.8 ± 8.4 (2–45.7)	13.6 ± 5.7 (4.5–29.9)	13.3 ± 6 (6–32)
Number of transfusions	146	93	60	63	34
Nonsplenectomized patients					
CCI (range)	15.5 ± 6.3 (4.1–32.4)	16.1 ± 5.9 (2.7–34.9)	13.6 ± 5.5 (2–31.1)	13 ± 5 (4.5–27.2)	13.2 ± 6 (6–32)
No. of Transfusions	135	81	53	60	33
Percentage of transfusions with CCI < 7.5	5%	3.7%	13.2%	15%	15%

*Mean ± SD × 10^{-3}.

Table 3. Corrected Count Increments (18- to 24-Hour)

	Duration of Storage (Days)				
	1	2	3	4	5
Total patients					
CCI	12.4 ± 6.7*	11.7 ± 6.7	11.3 ± 5.7	8.8 ± 4.6	8.9 ± 4.8
(range)	(0–31)	(1.7–33.5)	(2–26.6)	(2.9–22)	(3.2–20.1)
No. of transfusions	84	57	26	37	20
Nonsplenectomized patients					
CCI	10.9 ± 5.3	10.5 ± 5.5	10 ± 5.2	8.5 ± 4.4	8.9 ± 5
(range)	(0–28.1)	(1.7–33)	(2–26.6)	(2.9–22)	(3.2–20.1)
No. of transfusions	74	50	22	34	19

*Mean ± SD × 10^{-3}.

CCI of 12,200 (N = 16). All units in this earlier study had pH determinations done pretransfusion. Units with pH values of ≤ 6.0 (21% of the PC tested) were not transfused, and the figure of 12,200 is therefore an overestimate of what is likely to be achieved in more routine practice. Thus, the increments noted with the newer bags in the present study represent an important improvement over these earlier results. Bleeding times were not done in the current study, and we cannot comment on the relative hemostatic effectiveness of PC stored for differing durations. Small studies with platelets stored for five days have shown shortening of bleeding times after transfusion however,[1] and refractory bleeding was not a problem in any of the recipients in the current study.

These data also indicate that the pH of platelet concentrates can be uniformly and adequately maintained for the 5 days of storage. It has been shown that platelet viability deteriorates at a final pH of 6.0 to 6.2.[12] The lowest pH noted in this study was 6.9 despite an appreciable number of units with relatively high platelet bag counts and final platelet concentrations (Table 1). This is consistent with previous observations by Murphy et al[1] who noted a pH of <6.7 in only 1 of 101 units of PC stored for five days in PL-732 bags in volumes of 30 to 60 mL. In fact, the only concern may be that in some bags the pH might actually be higher than desirable because of increased diffusion of CO_2 (Table 1). Morphological changes and platelet clumping can be noted at these higher pHs.[13] It should be noted, however, that the mean PC volume of 65 mL in the present study is higher than the 50- to 55-mL volumes used in many blood centers. It may be that a higher pH will occur less frequently in these smaller preparations. Indeed, in the study by Murphy and colleagues[1] pH values of >7.4 were noted in only 7 of 101 units stored for five days. Nonetheless, there is legitimate concern about the potential for higher pH in PC with lower platelet counts, particularly after shorter periods of storage,[14] and further studies describing the in vivo correlates of these observations are necessary.

A potential concern about extension of platelet storage is the theoretical risk of increased bacterial growth in any unit contaminated inadvertently during collection.[15] Cultures were not obtained during the present study, and there were no posttransfusion episodes suggestive of infusion of bacterially contaminated platelets. Although more recent studies[16,17] have tended to minimize the concern raised by an earlier investigation describing episodes of transfusion of bacterially contaminated platelets,[18] clinicians should nonetheless be aware of the potential for this serious but fortunately rare complication.

The improvement in plastic bags for platelet storage has had a number of major impacts. First, the ability to extend storage to five days allows blood banks to stockpile platelets over holidays, long weekends, and other periods of donor shortage. This has become of even greater importance recently because of the further delays in distribution imposed by HTLV-III antibody testing. In addition, ~10% to 20% of units of platelet concentrate had unacceptably low pH after 72 hours of storage in the older bags,[4,12,13] and recipients of such units were exposed to the risks of transfusion with no chance of clinical benefit. The improvement in gas transport

Table 4. Ratio of 18- to 24-Hour Posttransfusion CCI/One-Hour Posttransfusion CCI

	Duration of Storage (Days)				
	1	2	3	4	5
Total patients					
Mean ± SD	0.7 ± 0.3*	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.6 ± 0.2
(range)	(0–1.8)	(0.1–1.0)	(0.2–1.1)	(0.2–1.1)	(0.3–1.0)
No. of transfusions	84	57	26	37	20
Nonsplenectomized patients					
CCI	0.7 ± 0.3	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
(range)	(0–1.8)	(0.1–1.0)	(0.2–1.1)	(0.3–1.1)	(0.3–1.0)
No. of transfusions	74	50	22	34	19

*Mean ± SD.

has eliminated these falls in pH even at higher bag platelet counts. Last, these technical advances have improved the quality of stored platelets at all points after collection and preparation. Most platelets are transfused after two to four days of storage, and the newer bags have further "guaranteed" the quality of such platelets. The data presented demonstrate that platelets stored for as long as five days in newer containers are clinically acceptable and will provide adequate transfusion support any time during their five-day storage period.

REFERENCES

1. Murphy S, Kahn RA, Holme S, Phillips GL, Sherwood W, Davisson W, Buchholz DH: Improved storage of platelets for transfusion in a new container. Blood 60:194, 1982

2. Snyder EL, Bookbinder M, Kakaiya R, Ferri P, Kiraly T: Five-day storage of platelet concentrates in CLX containers: Effect of type of agitation. Vox Sang 45:432, 1983

3. Rock G, Sherring VA, Tittley P: Five-day storage of platelet concentrates. Transfusion 24:147, 1984

4. Hogge DE, Thompson BW, Schiffer CA: Platelet storage for seven days in second generation CLX blood bags. Transfusion 26:131, 1986

5. Archur GT, Grimsley PG, Jindra J, Robson JE, Ribeiro A: Survival of transfused platelets collected into new formulation plastic packs. Vox Sang 43:223, 1982

6. Schiffer CA: What are the parameters to be controlled in platelet concentrates in order that they be offered to the medical profession as a standardized product with specific properties? Vox Sang 40:122, 1981

7. Filip DJ, Aster RH: Relative hemostatic effectiveness of human platelets stored at 4 °C and 22 °C. J Lab Clin Med 91:618, 1978

8. Slichter SJ, Harker LA: Preparation and storage of platelet concentrates. II. Storage variables influencing platelet viability and function. Br J Haematol 34:403, 1976

9. Schiffer CA: Platelet transfusion therapy for patients with cancer, in Higby DH (ed): Supportive Care of the Cancer Patient. Holland, Martinus Nijhoff Publishers, p 45, 1983

10. Hogge DE, Dutcher JP, Aisner J, Schiffer CA: Lymphocytotoxic antibody is a predictor of response to random donor platelet transfusion. Am J Hematol 14:363, 1983

11. Coller BS, Franza BR, Gralnick HR: The pH dependence of quantitative ristocetin induced platelet aggregation: Theoretical and practical implications—A new device for maintenance of platelet-rich plasma pH. Blood 47:841, 1976

12. Murphy S, Sayar SN, Gardner FH: Storage of platelet concentrates at 22 °C. Blood 35:549, 1970

13. Murphy S, Gardner FH: Platelet storage at 22 °C: Role of gas transport across plastic containers in maintenance of viability. Blood 46:209, 1978

14. Murphy S: Platelet storage for transfusions. Semin Hematol 22:165, 1985

15. Blajchman MA, Thornley JH, Richardson H, Eleder D, Spiak C, Racher J: Platelet transfusion-induced serratia marcescens sepsis due to vacuum tube contamination. Transfusion 19:39, 1979

16. Silver H, Sonnenwirth AC, Beisser LD: Bacteriologic study of platelet concentrates prepared and stored without refrigeration. Transfusion 10:315, 1970

17. Katz AJ, Tilton RC: Sterility of platelet concentrates stored at 25 °C. Transfusion 10:329, 1970

18. Buchholz DH, Young VM, Friedman NR, Reilly JA, Mardiney MR: Bacterial proliferation in platelet products stored at room temperature. Transfusion-induced enterobacter sepsis. N Engl J Med 285:429, 1971

Purification and Characterization of Human Platelet Glutathione-S-Transferase

By Joseph Loscalzo and Jane Freedman

A glutathione-S-transferase was isolated and purified to homogeneity from human platelets. With a combination of ammonium sulfate fractionation and chromatographic methods, 0.2 mg of pure enzyme was obtained from 9×10^{11} platelets with a 12% recovery. The purified enzyme had a specific activity of 7.5 U per milligram, representing an ~1,100-fold purification. The enzyme was found to be anionic, with an isoelectric point of 4.6. With reduced glutathione as a co-substrate, platelet glutathione-S-transferase was most active with the synthetic substrate, 1-chloro-2,4-dinitrobenzene, less active with 1,2-dichloro-4-nitrobenzene, and essentially inactive with nitroglycerin and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane. The pH optimum for activity with glutathione and 1-chloro-2,4-dinitrobenzene was 7.0. Indomethacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methyindole-3-acetic acid), a chlorobenzene derivative, noncompetitively inhibited human platelet glutathione-S-transferase with an apparent K_i of 0.23 mmol/L. This study represents the first complete purification and characterization of a glutathione-S-transferase from platelets. The presence of this enzyme in the platelet, within which high concentrations of reduced glutathione coexist, suggests the potential importance of the platelet in detoxification reactions and in the synthesis of the glutathione adducts of leukotriene metabolism.

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GLUTATHIONE-S-TRANSFERASES (GST) comprise a family of enzymes in humans that catalyze the conjugation of reduced glutathione (GSH) with various hydrophobic substrates bearing electrophilic centers.[1,2] These enzymes are dimers, are the products of three autosomal gene loci, and have been found in a number of isozymic forms in a variety of cell types, including liver, lung, placenta, and erythrocytes.[3-10] In light of data obtained mainly from experimental animals demonstrating the detoxifying function of these enzymes,[5] they may be important in determining a person's susceptibility to cancer.[11]

Platelets have recently been shown to contain GST activity by two groups of investigators,[12,13] but the enzyme(s) was incompletely characterized and only partially purified. Because (a) GSTs catalyze the conversion of the lipoxygenase pathway product, leukotriene A4, to the GSH adduct, leukotriene C4[14,15]; (b) platelets are rich in GSH (~1 to 2 mmol/L),[16-19] and repleting the store of GSH with cysteine results in enhanced leukotriene C4 production by (rabbit) platelets[20]; and (c) the platelet function inhibitor, indomethacin, a chlorobenzene derivative, has been shown to inhibit GST from liver,[21,22] we isolated to homogeneity a glutathione-S-transferase from human platelets, characterized its structural and enzymatic properties, and demonstrated that its activity may be inhibited noncompetitively by indomethacin.

MATERIALS AND METHODS

Materials. Carboxymethyl-cellulose (CM 52) and diethylaminoethanol (DEAE)-cellulose (DE-52) were purchased from Whatman Biochemicals, Ltd, Kent, England. Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Stockholm, Sweden. Ampholines were obtained from LKB Produkter, Bromma, Sweden. Glutathione-agarose (formed from the epoxide derivative of agarose and linked to glutathione through its sulfur atom) and 1,2-epoxy-3-(*p*-nitrophenoxy)-propane (ENPP) were obtained from Sigma Chemical Co, St. Louis. Centriflo membrane cones (CF 25) were obtained from Amicon Corp, Danvers, Mass. GSH was purchased from Calbiochem-Behring, San Diego. 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), sulfanilamide, and *N*-(1-naphthyl)ethylenediamine diHCl were obtained from Aldrich Chemical Co, Milwaukee. Nitroglycerin was purchased from Marion Laboratories, Kansas City, Mo. All other chemicals used were reagent grade or better. Deionized water was used throughout.

Platelets. Platelets were obtained from the Massachusetts Affiliate of the American Red Cross in 10- to 20-unit lots that were 48 to 72 hours old. The platelets were stored as platelet-rich plasma (PRP) in acid-citrate-dextrose (NIH Formula A) at room temperature before use. Concentrates from single donors routinely contained $\sim 2 \times 10^{11}$ platelets per 100 mL of plasma.

Activity measurements. During purification, enzyme activity was monitored by measuring the conjugation of CDNB with GSH.[3] The standard 1-mL assay contained 1 mmol/L of GSH and 1 mmol/L of CDNB in 0.1 mol/L of potassium phosphate, pH 6.5. Varying amounts of enzyme (in 20- to 500-μL aliquots) were added to start the reaction, and the rate of increase in absorbance at 340 nm was monitored at 25 °C for one to five minutes. During this period, the reaction rate was linear with time, as well as with respect to enzyme concentration. One unit of enzymatic activity is defined as that forming 1 μmol of product per minute at 25 °C. The molar extinction coefficient of the CDNB-GSH adduct was 9,600 mol/$L^{-1}cm^{-1}$.

Enzyme activity using DCNB was assessed by measuring its conjugation with GSH at pH 7.5. Changes in absorbance at 345 nm were monitored at 25 °C on addition of enzyme to a 1 mmol/L of solution of DCNB and 5 mmol/L of GSH in 0.1 mol/L of potassium phosphate.[3] The molar extinction coefficient of the DCNB-GSH adduct was 8,500 mol/$L^{-1}cm^{-1}$. Adduct formation using 5 mmol/L of ENPP and 5 mmol/L GSH at pH 6.5 was monitored similarly at 25 °C in 0.1 mol/L of potassium phosphate by following changes in absorbance at 360 nm, using a molar extinction coefficient for the adduct of 500 $(mol/L)^{-1}\ cm^{-1}$.[3]

Denitrification of nitroglycerin was measured using the assay of Snell and Snell[23] involving diazotization of sulfanilic acid and subsequent coupling with the chromophore, *N*-(1-naphthyl)ethylenediamine, as described by Habig and colleagues.[3]

Gel filtration. The mol wt of platelet GST was estimated on a calibrated column (30 × 1.2 cm) of Sephadex G-75 in 0.1 mol/L of

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potassium phosphate, pH 7.0, with 5 mmol/L of GSH. Fractions (0.5 mL) were collected, and the mobility of GST was compared with that of reduced von Willebrand protein (mol wt 230,000, defining the void volume), hen egg white lysozyme (mol wt 14,400), human myoglobin (mol wt 18,000), hen egg white ovalbumin (mol wt 45,000), and bovine serum albumin (BSA) (mol wt 66,200).

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Weber and Osborne[24] and modified by Laemmli.[25] Protein samples were mixed with three volumes of 0.06 mol/L of Tris-HCl, pH 6.8, 3% SDS, and 0.5% 2-mercaptoethanol. Fourteen percent slab gels were polymerized with a ratio of acrylamide to methylene bisacrylamide of 37:1. The gels were stained with 0.25% Coomassie brilliant blue in 50% methanol and 5% acetic acid and destained by diffusion. Molecular weight standards were processed similarly and included hen egg white lysozyme (mol wt 14,400), soybean trypsin inhibitor (mol wt 21,500), carbonic anhydrase (mol wt 45,000), BSA (mol wt 66,200), and phosphorylase B (mol wt 92,500).

Preparative isoelectric focusing. The isoelectric point of platelet GST was determined by electrofocusing in a sucrose density gradient with a 110-mL column (LKB) using 1% Ampholine in the pH 3.0 to 10.0 range. Fractions of 0.5 mL were collected after 72 hours at 300 V.

Amino acid analysis. The amino acid composition of purified platelet GST was performed by high-performance liquid chromatography using a PicoTag System (Waters Associates, Milford, Mass).

Protein determinations. Protein concentrations were determined by the method of Lowry and colleagues.[26]

RESULTS

Purification of human platelet GST. All procedures and yields are based on 20 U of PRP as a starting material. The pH was adjusted to 6.5 with acid-citrate-dextrose (NIH Formula A) and the platelets were separated from residual erythrocytes by centrifugation at 180 g for 20 minutes, then washed three times with 10 mmol/L of Tris-HCl, pH 7.8, 0.15 mol/L of NaCl, 10 mmol/L of EDTA, and 1.4 mmol/L of 2-mercaptoethanol. Washed platelets were disrupted by exposure to 1,000 psi of nitrogen for 20 minutes, after which the insoluble residue was sedimented at 35,000 g for 50 minutes. To the supernatant (which contained all of the enzymatic activity) was added 16.4 g of solid ammonium sulfate per 100 mL at 4 °C. The precipitate was collected by centrifugation at 17,000 g for 35 minutes, and the supernatant was saved. To the supernatant was added 40.2 g of solid ammonium sulfate per 100 mL at 4 °C and the precipitate was collected similarly. The ammonium sulfate pellet was resuspended in 5 mmol/L of potassium phosphate, pH 7.0, 1.4 mmol/L of 2-mercaptoethanol, and was dialyzed extensively at 4 °C against the same buffer to remove ammonium sulfate.

After dialysis, the solution was applied to a CM-52 column (24 x 2 cm) at 4 °C and eluted with 5 mmol/L of potassium phosphate, pH 7.0, 1.4 mmol/L of 2-mercaptoethanol. Similar attempts at isolating a cationic isozyme from a DE-52 column failed to demonstrate any activity with low ionic strength elution buffers. Twenty 5-mL fractions were collected from the CM-52 column at 55 mL per hour with the most active fractions found in tubes 8 and 9. These fractions were dialyzed against 20 mmol/L of potassium phosphate, pH 7.0, at 4 °C and subsequently applied to a glutathione-agarose column (16 x 1.2 cm) in the same buffer. GST was eluted at 10 mL per hour with 50 mmol/L of Tris, pH 9.6 and 5 mmol/L of GSH. The eluted GST was concentrated in a CF 25 Centri-cone and applied to a Sephadex G-75 column (30 x 1.2 cm) for final purification and mol wt estimation. This column was developed with 0.1 mol/L of potassium phosphate, pH 7.0, and 5 mmol/L of GSH. Fractions (0.5 mL) were collected at a flow rate of 10 mL per hour.

A summary of the results of purification is shown in Table 1. Based on the specific activity of the homogeneous enzyme, purified ~1,000-fold with a 12% recovery, it may be estimated that ~1.7 mg of GST is present in 9×10^{11} platelets.

Homogeneity and mol wt determinations of platelet GST. The isolated enzyme appeared to be homogeneous by two criteria. It eluted from a Sephadex G-75 column as a single coincident peak of activity and protein concentration (Fig 1). In addition, SDS-PAGE of the purified enzyme in the presence of 2-mercaptoethanol revealed a single band (Fig 2).

The mol wt of the native, intact molecule was estimated to be 48,000 daltons by Sephadex G-75 chromatography, assuming a shape and partial specific volume identical to that of the standards listed in Materials and Methods. Subunit mol wt was determined from SDS-PAGE in the presence of 2-mercaptoethanol and found to be ~25,000 daltons.

Determination of isoelectric point. Isoelectric focusing of the purified enzyme was performed as described in Materials and Methods. Essentially all of the activity was present in a single tube at a pH of 4.60 (Fig 3).

Amino acid analysis. The amino acid composition of the purified enzyme is shown in Table 2.

Substrate specificity and pH optimum. The substrate specificity of platelet GST is indicated in Table 3. The

Table 1. Purification of Glutathione-S-Transferase From Human Platelets

	Vol (mL)	Protein (mg)	Activity (U)	Specific Activity (U/mg)	Purification (-fold)
Disrupted platelets	44	1846	12.92	0.007	—
Disrupted platelet supernatant	40	485	12.77	0.026	3.7
Ammonium sulfate fraction (80%)	10	82	5.17	0.063	9.0
CM-cellulose (CM-52)	10	39	5.07	0.13	18.6
Affinity chromatography	0.5	0.42	1.56	3.72	531.4
Sephadex G-75	0.5	0.20	1.50	7.50	1071.4

These data were derived from preparations starting with ~9×10^{11} platelets as described in the Materials and Methods section.

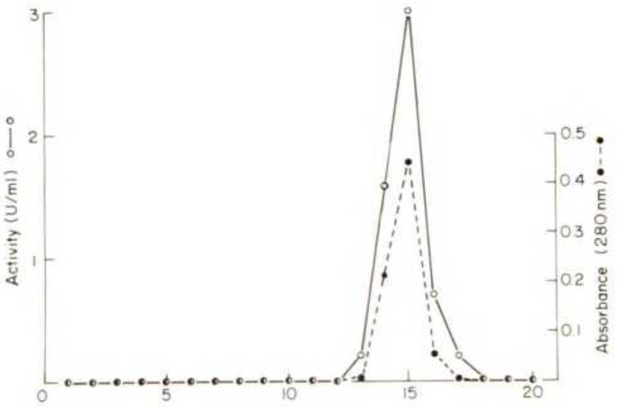

Fig 1. Sephadex G-75 chromatography of human platelet glutathione-S-transferase (GST). Purified GST was applied to a 30 × 1.2 cm column of Sephadex G-75 at 4 °C in 0.1 mol/L of potassium phosphate, pH 7.0, 5 mmol/L of reduced glutathione (GSH). Fractions (0.5 mL) were collected at 10 mL per hour. Activity (○) was assessed as described in the Materials and Methods section and plotted with absorbance at 280 nm (●).

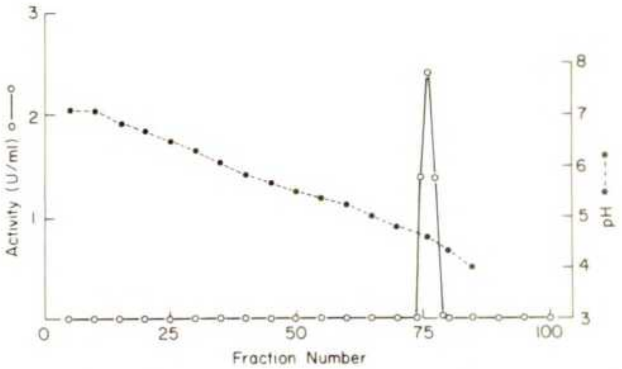

Fig 3. Isoelectric focusing of human platelet glutathione-S-transferase (GST). Fractions (0.50 mL) were collected, and the pH (●) and activity (○) were measured (as described in the Materials and Methods section).

platelet enzyme is most active with CDNB, is less so with DCNB, and is essentially inactive with nitroglycerin and ENPP, all with reduced glutathione as co-substrate. The K_m and V_{max} values with the standard substrate, CDBN, were 0.65 mmol/L and 312 μmol per minute per micromole of protein, respectively. GSH was an essential second substrate in the assay with CDNB, in that cysteine, *N*-acetylcysteine, 2-mercaptoethanol, and dithiothreitol were completely inactive as substitutes. Using 1 mmol/L of CDNB as a co-substrate, the K_M of GST for GSH was 0.12 mmol/L. Examining enzymatic activity from pH 4.0 to 9.0 demonstrated that the pH optimum was 7.0 for CDNB and GSH.

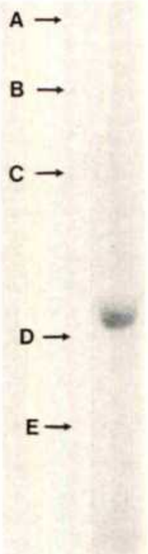

Fig 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified human platelet glutathione-S-transferase (GST). Fourteen percent slab gels were used, and the sample was prepared with 3% SDS and 0.5% 2-mercaptoethanol. Molecular weight standards include: (A) phosphorylase B (mol wt 92,500); (B) bovine serum albumin (mol wt 66,200); (C) carbonic anhydrase (mol wt 45,000); (D) soybean trypsin inhibitor (mol wt 21,500); (E) hen egg white lysozyme (mol wt 14,400).

Inhibition of GST activity by indomethacin. Indomethacin (1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid), an inhibitor of prostaglandin synthesis, inhibited human platelet GST in a noncompetitive manner (Fig 4) with respect to DCNB. The apparent K_I was 0.23 mmol/L and the apparent K_M was 0.65 mmol/L. Acetylsalicylic acid failed to inhibit the enzyme at concentrations up to 400 μmol.

DISCUSSION

We report that platelets contain an anionic GST which may be readily isolated and purified ~1,100-fold with a 12% recovery by a combination of ammonium sulfate fractionation and chromatographic techniques. The kinetic parameters that define its activity with well-characterized synthetic

Table 2. Amino Acid Composition of Glutathione-S-Transferase From Human Platelets

Amino Acid	Residues (per mol)
Lys	25.1
His	5.5
Arg	20.2
Asx	43.3
Thr	19.1
Ser	28.0
Glx	53.4
Pro	25.8
Gly	50.1
Ala	36.0
Cys	5.1
Val	28.2
Met	10.0
Ile	12.1
Leu	52.2
Tyr	18.1
Phe	13.2

Hydrolysis was performed in boiling HCl for 48 hours.

Table 3. Substrate Specificity of Human Glutathione-S-Transferase

Substrate	Specific Activity (µmol per minute per µmol)
1-chloro-2,4-dinitrobenzene	156
1,2-Dichloro-4-nitrobenzene	62
Nitroglycerin	0.13*
1,2-Epoxy-3-(*p*-nitrophenoxy)-propane	0.63*

Standard assay conditions are described in the Materials and Methods section.

*Because no enzyme activity was observed, this value represents the lower limit of the assay at the highest enzyme concentration used.

substrates and GSH suggest that it is different from the liver transferases.[4-7] Two lines of evidence argue that platelet GST is not identical to the erythrocytic enzyme. First, there is significantly more methionine and isoleucine in platelet GST than erythrocyte GST.[10] Second, the V_{max} of platelet GST is 0.07 that of erythrocyte GST, and the ratio of activities toward CDNB and DCNB (CDNB/DCNB) is 2.5 for platelet GST and 2640 for erythrocyte GST.[10]

The possibility that erythrocyte contamination might contribute significantly to total isolated enzyme in the preparation of GST from platelets is quite unlikely since only 0.12 mg of GST may be isolated from 9 × 10^{11} erythrocytes,[10] whereas 0.2 mg is readily isolated from 9 × 10^{11} platelets in our experiments. With a contamination ratio of no more than one erythrocyte to each 3,000 platelets in our starting material, <0.02% of total enzyme isolated would be provided by erythrocytes.

The presence of GST activity in platelets suggests the possibility that platelets may have a role in detoxification reactions in plasma or that lipoxygenase activity in these cells may lead to the formation of the glutathione adduct of leukotriene A4, leukotriene C4, as originally suggested by two groups of investigators.[20,27] Because of the controversy regarding the presence of leukotriene C4 in platelets and its role in platelet function,[28-31] another possible explanation for the presence of this potent enzyme in the platelet may simply be that it serves as a site for leukotriene A4 conversion to leukotriene C4 using leukotriene A4 provided by *other* cells, such as leukocytes or endothelial cells. Precedent for such intercellular shuttling of prostanoid intermediates exists[32,33] between platelets and leukocytes and, given the high intracellular concentration of GSH in platelets (as great as that found in erythrocytes), the platelet may serve as a sink for GST co-substrate provided by cells with less GSH. The low K_M of GST for GSH (using CDNB as a co-substrate) notwithstanding, Bach and colleagues[34] have shown that 1.0 mmol/L of GSH is required for optimal activity of GST (isolated from rat basophilic leukemia cells) with leukotriene A4 as a co-substrate.

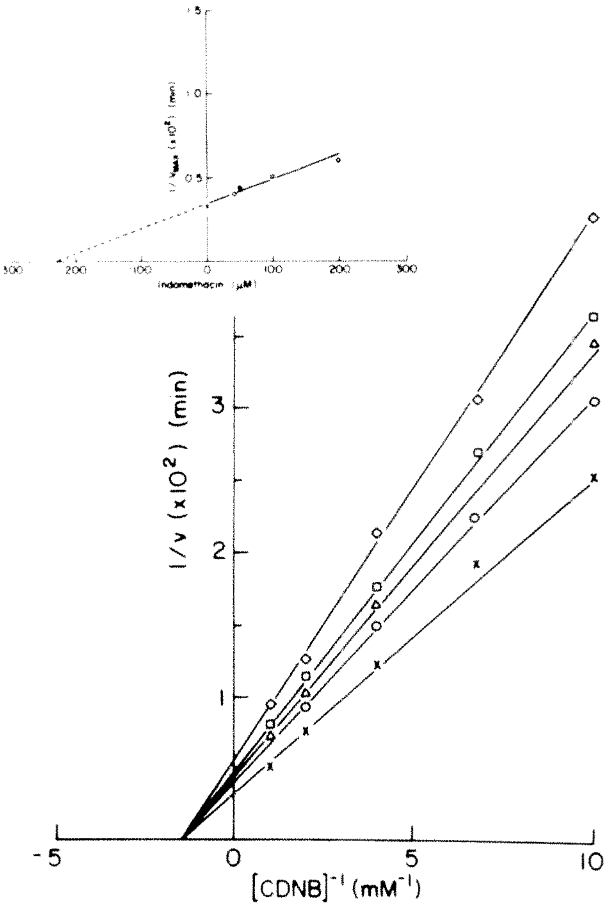

Fig 4. Inhibition of platelet glutathione-S-transferase (GST) by indomethacin. Activity of GST was measured varying both 1-chloro-2,4,-dinitrobenzene (CDNB) and indomethacin concentrations. Assays were performed at 25 °C in 0.1 mol/L of potassium phosphate, pH 6.5, 1 mmol/L of reduced glutathione (GSH). Twenty-microliter aliquots of enzyme were added to start the reaction, and the increase in absorbance at 340 nm was monitored for one to five minutes. The indomethacin concentrations used were 0 (×), 40 µmol/L (○), 50 µmol/L (△), 100 µmol/L (□), and 200 µmol/L (◇). Points represent the mean of duplicates. The inset depicts a plot of $1/V_{max}$ (derived from the *y*-intercept of the double-reciprocal plot) as a function of indomethacin concentration, by which graphic method the apparent K_i may be determined.

The inhibition of platelet GST by indomethacin supports the suggestion by Nicholls and Ahokas[22] using liver isozymes of GST that the lower the isoelectric point, the more effective is inhibition by indomethacin. In addition, the inhibition of platelet GST activity suggests that if leukotriene C4 production is an important function of platelets, inhibition at this step may serve as a significant control point in arachidonate metabolism in these cells. Indomethacin has already been shown to inhibit prostaglandin synthetase,[33] as well as 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase.[34] That GST is also inhibited by this agent firmly supports the hypothesis that indomethacin has a direct effect on both the cyclooxygenase and lipoxygenase pathways. In the platelet, the extent to which each pathway may be affected by a given concentration of indomethacin depends on the relative concentrations of the prostaglandin synthetase, the peroxidase, and GST, and the K_I values for each of these enzymes with respect to physiologic substrates. Studies in vitro suggest that the concentration of indomethacin required for inhibition of cyclooxygenase is lower than that reported here for GST inhibition.

The true in vivo pharmacologic importance of platelet GST inhibition by indomethacin in terms of its inhibition of leukotriene C4 synthesis has yet to be determined. That the inhibitory mechanism is noncompetitive suggests that, despite the major structural differences between CDNB and leukotriene A4 as substrates for GST, inhibition may well be important for the latter substrate as well.

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REFERENCES

1. Booth J, Boyland E, Sims P: An enzyme from rat liver catalyzing conjugations with glutathione. Biochem J 79:516, 1961
2. Boyland E, Chassequd LF: The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. Adv Enzymol Rel Areas Mol Biol 32:172, 1969
3. Habig WH, Pabst MJ, Jakoby WB: Glutathione-S-transferase—The first enzymatic step in mercapturic acid formation. J Biol Chem 249:7130, 1974
4. Kamisaka K, Habig WH, Ketley JN, Arias IM, Jakoby WB: Multiple forms of human glutathione-S-transferase and their affinity for bilirubin. Eur J Biochem 60:153, 1975
5. Jakoby WB: The glutathione-S-transferases—A group of multifunctional detoxification proteins. Adv Enzymol Rel Areas Mol Biol 46:383, 1978
6. Awasthi YC, Dao DD, Saneto RP: Interrelationship between anionic and cationic forms of glutathione-S-transferases of human liver. Biochem J 191:1, 1980
7. Warholm M, Guthenberg C, Mannervik B: Molecular and catalytic properties of glutathione transferase μ from human liver—An enzyme efficiently conjugating epoxides. Biochemistry 22:3610, 1983
8. Mannervik B, Jensson H: Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione S-transferases in rat liver cytosol. J Biol Chem 257:9909, 1982
9. Dao DD, Partridge CA, Kurosky Q, Awasthi YC: Human glutathione-S-transferase—Characterization of the anionic forms from lung and placenta. Biochem J 221:33, 1984
10. Marcus CJ, Habig, WH, Jakoby WB: Glutathione transferase from human erythrocytes. Arch Biochem Biophys 188:287, 1978
11. Board PG: Biochemical genetics of glutathione-S-transferase in man. Am J Hum Gen 33:36, 1981
12. Hofmann J, Hofmann B, Sturm G, Till U: Evidence for glutathione-S-transferase activity in human blood platelets. Biomed Biochim Acta 42:K5, 1983
13. Rogerson KS, Mitchell D, Lawton A, Ibbotson R, Cotton W, Strange RC: Studies on the glutathione-S-transferase of human platelets. Biochem Biophys Res Commun 122:407, 1984
14. Hammarstrom S, Murphy RC, Samuelsson B: Structure of leukotriene C—Identification of the amino acid part. Biochem Biophys Res Commun 91:1266, 1979
15. Parker CW, Huber MM, Hoffman MK, Falkenhein SF: Characterization of the two major species of slow reacting substance from glutathionyl ethers of eicosatetraenoic acids oxygenated at the 5 position. Prostaglandins 18:673, 1979
16. Ando Y, Steiner M, Baldini MG: Effect of storage on sulfhydryl and disulfide groups in human platelets. Blood 43:121, 1974
17. Matsuda S, Ikeda Y, Aoki M, Toyama K, Watanabe K, Ando Y: Role of reduced glutathione on platelet function. Thromb Haemost 42:1324, 1979
18. Hofmann J, Losche W, Till W, Bosia A, Arese P: Effect of decreased GSH level on human platelet functions. Artery 8:431, 1980
19. Loscalzo J: *N*-Acetylcysteine potentiates inhibition of platelet function by nitroglycerin. J Clin Invest 76:703, 1985
20. Meringolo C, Bartolini G, Orlandi M, Tomasi V, Barna O: Is a slow-reacting substance-like compound involved in rat platelet aggregation? Prostaglandins Med 2:381, 1979
21. Wu C, Matthews KP: Indomethacin inhibition of glutathione-S-transferases. Biochem Biophys Res Commun 112:980, 1983
22. Nicholls FA, Ahokas JT: Inhibition of purified glutathione-S-transferases by indomethacin. Biochem Biophys Res Commun 119:1034, 1984
23. Snell FD, Snell CJ: Colorimetric Methods of Analysis, 3rd ed, Vol 2. New York, Van Nostrand, 1949, p 804
24. Weber K, Osborne M: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406, 1969
25. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature London 227:680, 1970
26. Lowry OH, Rosebrough NJ, Farr AL, Randall R: Protein measurement with the Folin reagent. J Biol Chem 193:265, 1951
27. Mencia-Huerta JM, Hadji L, Benveniste J: Release of a slow-reacting substance from rabbit platelets. J Clin Invest 68:1586, 1981
28. Sams AR, Sprecher HS, Sankarappa SK, Needleman P: Selective inhibitors of platelet arachidonate metabolism—Aggregation independent of lipoxygenase, in Samuelsson B, Paoletti R (eds): Leukotrienes and Other Lipoxygenase Products. New York, Raven, 1982, p 19
29. Schafer A: Deficiency of platelet lipoxygenase activity in myeloproliferative disorders. N Engl J Med 306:381, 1982
30. Dutilh CE, Haddeman E, Don JA, Ten Hoor F: The role of arachidonate, lipoxygenase, and fatty acids during irreversible blood platelet aggregation in vitro. Prostaglandins Med 6:111, 1981
31. Dutilh CE, Haddeman E, Jouvenaz GH, Ten Hoor F, Nugteren DH: Study of the two pathways for arachidonate aggregation in blood platelets. Lipids 14:241, 1978
32. Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ: Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest 66:979, 1980
33. Schafer AI, Crawford DD, Gimbrone MA Jr: Unidirectional transfer of prostaglandin endoperoxides between platelets and endothelial cells. J Clin Invest 73:1105, 1984
34. Bach MK, Brashler JR, Morton DR, Steel LK, Kaliner MA, Hugli TE: Formation of leukotrienes C and D, and pharmacologic modulation of their synthesis, in Samuelsson B, Paoletti R (eds): Leukotrienes and Other Lipoxygenase Products. New York, Raven, 1982, p 103
35. Flower RJ, Vane JR: In Robinson HJ, Vane JR (eds): Prostaglandin Synthetase Inhibitors. Some Pharmacologic and Biochemical Aspects of Prostaglandin Biosynthesis and its Inhibition. New York, Raven, 1974, p 9
36. Siegel MI, McConnell RT, Cuatrecasas P: Aspirin-like drugs interfere with arachidonate metabolism by inhibition of the 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid peroxidase activity of the lipoxygenase pathway. Proc Natl Acad Sci 76:3774, 1979

Lymphocyte Subsets in Normal Bone Marrow

By Pamela Clark, David E. Normansell, Donald J. Innes, and Charles E. Hess

Bone marrow aspirates and biopsies from ten normal donors were stained directly with monoclonal antibodies specific for lymphocyte, monocyte, and myeloid antigens, and were analyzed by flow cytometry. To avoid cell loss, lymphocytes were not specifically isolated prior to staining. T cells comprised 46% of aspirate lymphocytes and 22% of biopsy lymphocytes. Further, the Leu-3:Leu-2 ratio of bone marrow T cells was below 1.0. B cells comprised 8% to 11% of bone marrow lymphocytes in both aspirates and biopsies, and there was a substantial percentage of cells in the lymphocyte window that was negative for all B and T cell markers. The lymphocyte window had very little myeloid contamination; however, when the myeloid window was examined, staining was greater than 90%.
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BONE MARROW studies are an important part of the workup for many hematologic abnormalities, particularly the acute leukemias. Our understanding of these diseases has increased dramatically with the data provided by the monoclonal antibodies to lymphocyte subsets. However, no clear definition of lymphocyte subsets in normal bone marrow is available. First, analysis has often been performed on marrow from patients who, although without hematologic malignancy, were not clinically normal.[1-5] Second, most past studies isolated the lymphocytes from the marrow sample using various density separation media,[1-8] with no guarantee that the cells obtained were representative of the whole tissue. Third, most studies were performed prior to the advent of monoclonal antibodies, limiting the accuracy, number, and cell types capable of being identified. In addition, marrow aspirate specimens are frequently contaminated by peripheral blood,[5] resulting in equivocal findings. This study was designed to circumvent these problems. We obtained clinically normal donors and did not separate the lymphocytes. Lymphocytes were labeled with monoclonal antibodies and analyzed on the flow cytometer. Flow cytometry can easily isolate lymphocytes from a heterogeneous cell population based on their size and granularity without the necessity of purification. Once the lymphocyte population has been determined, the various percentages of lymphocytes marking with monoclonal antibodies can be calculated. Both aspirate and biopsy specimens were used in our study. An analysis of biopsy specimens served as a control to the aspirate, the common means of sampling.

MATERIALS AND METHODS

All studies were approved by the Human Investigation Committee of the University of Virginia Hospital. After informed consent had been obtained, samples were drawn from ten, paid, normal, adult volunteers, whose ages ranged from 24 to 42 years. Except for birth control pills, none of the donors was taking medications. Specimens obtained from each volunteer consisted of a peripheral blood sample, a bone marrow aspirate, and a solid bone marrow biopsy core. The three specimen types were analyzed with the following battery of monoclonal antibodies: for B cells, B-4 (Coulter, Inc, Hialeah, Fla) and Leu-12 (Becton Dickinson, Inc, Mountain View, Calif); and for T cells and the various T cell subsets, Leu-1, Leu-4, Leu-3 and Leu-2 (Becton Dickinson). The thymocyte marker T-6 (Ortho Diagnostics, Raritan, NJ) was also used to detect any T cell precursors. Myeloid cells were examined using Leu-M1 and Leu-M3 (Becton Dickinson). In addition, the following antibodies were used: Leu-11 (Becton Dickinson), a natural killer (NK) cell marker; J-5 (Coulter) a common acute lymphocytic leukemia antigen (CALLA)-specific marker; and Ia (Ortho) a marker which stains a diverse population of cells including B cells, activated T cells, and stem cells. With the exception of Leu-M3, all antibodies were indirect. All tests, except B-4, were performed using 5 μL of monoclonal antibody; B-4 required 20 μL. The secondary antibodies used were fluorescein (FITC)-conjugated goat antimouse IgG_1 for Leu-2, Leu-3, Leu-4, Leu-12, and B-4; anti-IgG_2 for Leu-1, Ia, T6, and J-5; and anti-IgM for Leu-M1, and Leu-11 (Meloy Laboratories, Inc, Springfield, Va). These secondary antibodies were diluted 1:10 in RPMI 1640 (Whittaker MA Bioproducts, Walkersville, Md).

Four negative controls, one for each secondary antibody and a direct antibody control for Leu-M3 were also included in the test battery.

Peripheral blood. Approximately 3 mL of blood were obtained from an antecubital vein and placed in a 3-mL vacutainer tube containing EDTA. White cell counts (WBCs) were obtained using the Coulter ZBI. Peripheral blood smears were then prepared and stained with Wright's stain for later analysis.

Monoclonal antibody staining was performed using the Coulter whole blood procedure. One hundred microliters of peripheral blood were placed in each of 16 labeled plastic test tubes. The appropriate monoclonal antibody or phosphate-buffered saline (PBS) was added, and the cells were incubated on ice for ten minutes. The cells were then washed twice with 4 mL of Coulter PBS followed by centrifugation at 300 g for three minutes. Following the addition of 100 μL of the appropriate FITC-labeled secondary antibody, the cells were incubated on ice for ten minutes and washed twice. The red blood cells (RBCs) in each tube were then lysed using 1 mL of lysing reagent prepared by adding Coulter Immunolyse concentrate to Coulter PBS at a 1:25 dilution. After incubation in the lysing reagent for 30 seconds, 250 μL of Coulter fixative was added to each tube and the cells were immediately centrifuged at 300 g for three minutes. The supernatant was removed, and the cells were washed twice and resuspended in ~1 mL of Coulter PBS. Samples were then analyzed using an Epics-C flow cytometer (Coulter) set at 488 nm.

Bone marrow aspirates. Approximately 1 to 1.5 mL of bone marrow aspirate was removed from the left posterior iliac crest using a 15-gauge Jamshidi needle (American Pharmaseal, Valencia, Calif) and syringe. The specimen was immediately placed in a 3-mL vacutainer tube containing EDTA. White cell counts were performed directly on the specimen using the Coulter ZBI. Slides of the aspirate material were prepared, stained with Wright's stain, and differential counts were performed.

The remaining aspirate was stained with monoclonal antibodies

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using the Coulter whole blood procedure. The procedure was identical to that used for peripheral blood except that the amount of material used per test varied with the cell count. For aspirates with WBCs >50,000 per microliter, 25 μL of specimen were used. The amount of aspirate used per test was increased to 50 μL when cell counts were <50,000 per microliter.

Two-color analysis. Three aspirate specimens (donors 1, 6, and 7) were triple-labeled using Leu-M1 plus FITC secondary antibody, Leu-1 conjugated with phycoerythrin (PE) (Becton Dickinson) and Leu-12 conjugated with PE (a gift from Dr Christopher Snow, Becton Dickinson Monoclonal Antibody Center, Mountain View, Calif). Depending on the cell count, either 25 μL or 50 μL of aspirate were aliquotted into a plastic test tube. Five microliters of Leu-M1 were added, and the cells were incubated for 20 minutes on ice. The cells were then washed twice as described above. One hundred microliters of IgM secondary antibody solution was added, and the cells were again incubated for 20 minutes on ice and washed twice. Blocking was performed using 100 μL of 1% mouse serum diluted in PBS. The cells were incubated for 20 minutes and washed twice. After washing, 15 μL of Leu-12 PE and 10 μL of Leu-1 PE were added and the cells were incubated for 20 minutes on ice followed by two washes. Lysis and further washing were performed as in the aspiration procedure. Red and green fluorescence was then evaluated on the Epics-C at a setting of 488 nm.

Biopsy cores. Biopsy cores were obtained from the left posterior iliac crest using an 11-gauge Jamshidi needle. The cores were ≥2 cm in length. A small portion of the core was immediately placed in formalin for histologic processing, and the remainder was placed in a Petri dish with 5 mL of 50% Hanks balanced salt solution without calcium and magnesium (HBSS) (GIBCO Laboratories, Grand Island, NY) and 50% PBS. The core was gently teased apart with 23-gauge needles to obtain a unicellular suspension and was decanted into a conical centrifuge tube. The Petri dish was rinsed with 5 mL of 50% HBSS–PBS, and the rinse solution plus any remaining pieces of core were placed in a Potter-Elvehjem tissue grinder (Fisher Scientific, Silver Spring, Md). After gentle manipulation with the tissue grinder, the rinse solution was added to the cellular suspension and centrifuged at 300 g for ten minutes. RBCs in the cell pellet were lysed by adding 2 mL of ammonium chloride lysing reagent (8.29 g NH_4Cl, 1.0 g $KHCO_3$ to each liter of distilled water) and incubated on ice for ten minutes. The cells were harvested by centrifugation at 300 g for five minutes, the lysing reagent was aspirated, and the cells were washed twice in 10 mL of 50% HBSS–PBS. The cell pellet was then resuspended in 1 mL of HBSS–PBS solution. Cell counts were obtained using trypan blue dye to assess viability.

The suspension was adjusted to 5×10^6 to 1×10^7 cells per milliliter, and 100 μL of the cell suspension was added to each of 16 test tubes. The appropriate amount of monoclonal antibody was added, and the cells were incubated on ice for 30 minutes. After incubation, 2 mL of ice-cold PBS with 0.1% azide was added to each test tube, and the cells were recovered by centrifugation (ten minutes, 300 g) at 4 °C. The cells were again washed in 2 mL of PBS with 0.1% azide and centrifuged again. After the cells were washed, 100 μL of the secondary antibody was added, and the cells were incubated for 30 minutes on ice and protected from light. The cells were again washed twice and analyzed on the Epics-C at a setting of 488 nm.

Phenotypic analysis. All specimens were analyzed on the day of collection. Peripheral blood was examined first to determine the instrument settings that best separated the lymphocyte population from monocytes and granulocytes. The lymphocytes were then analyzed for the percentage of cells positive for each of the cell markers.

Analysis of the aspirate specimens generally required only slight modifications of the settings established for peripheral blood. To demonstrate that the majority of cells analyzed were lymphocytes, two-color analysis was performed on three aspirates. The percentage of cells staining simultaneously with FITC and PE was then compared to the values obtained from single fluorescence studies. In addition, the myeloid cells were separately examined, and the percentage staining with Leu-M1 and Leu-M3 was determined.

Analysis of biopsy specimens required changing the fluorescence settings so that clear isolation of the lymphocyte population could be obtained. High negative backgrounds caused by nonspecific staining of cellular debris were responsible for the setting changes. As with aspirates, myeloid cells were separately analyzed for staining with Leu-M1 and Leu-M3.

Table 1. Cell Marker Studies on Peripheral Blood of Ten Normal Donors: Cells (%) in Lymphocyte Window Positive for Specific Cell Surface Antigens

					B Cells		T Cells					Other			Myeloid	
					CD-19		CD-3	CD-5	CD-4	CD-8	CD-1	CD-16	CD-10		CD-15	
Donors	Sex	Age	WBC Ct × 10^3/μL	Lymphs (%)*	B-4	L-12	L-4	L-1	L-3	L-2	T-6	L-11	J-5	Ia	L-M1	L-M3†
1	M	33	5.3	46	6	8	84	79	47	38	0	2	5	10	0	ND
2	M	34	7.2	42	8	7	73	70	35	32	0	0	3	13	0	0
3	M	24	5.4	37	4	3	76	79	35	22	0	7	1	1	0	0
4	M	28	4.9	47	4	4	69	73	42	27	1	14	2	5	2	1
5	M	35	4.7	38	9	10	65	63	30	28	0	4	1	11	1	0
6	F	33	8.3	34	13	7	ND	64	40	33	0	0	2	6	1	10
7	F	42	8.7	25	3	4	ND	73	50	27	1	6	7	8	7	3
8	F	37	5.8	45	17	17	58	66	32	21	9	9	3	24	1	0
9	F	30	3.3	35	4	4	81	80	58	18	0	0	0	8	0	0
10	F	31	4.9	43	3	4	83	84	40	30	0	3	1	6	5	3
Average					7.1	6.8	73.6	73.1	40.9	27.6	1.1	4.5	2.5	9.2	1.7	1.9
Average for male donors					6.2	6.4	73.4	72.8	37.8	29.4	0.2	5.4	2.4	8.0	0.6	0.2
Average for female donors					8.0	7.2	74.0	73.4	44.0	25.8	2.0	3.6	2.6	10.4	2.8	3.2

ND, not done.

Determined by indirect immunofluorescence analysis using monoclonal antibodies and automated flow cytometry. Average negative background was: IgG_1, 4.0; IgG_2, 3.2; IgM, 5.8.

*% Lymphocyte percentages determined by examination of peripheral blood smears and performing differential.

†L-M3 used direct immunofluorescence; background was 1.2.

Table 2. Cell Marker Studies on Bone Marrow Aspirates From Ten Normal Donors: Cells (%) in Lymphocyte Window Positive for Specific Cell Surface Antigens

				B Cells		T Cells					Other			Myeloid	
				CD-19											
		Lymphs	WBC Ct			CD-3	CD-5	CD-4	CD-8	CD-1	CD-16	CD-10		CD-15	
Donors	Sex	(%)	× $10^3/\mu L$	B-4	L-12	L-4	L-1	L-3	L-2	T-6	L-11	J-5	Ia	L-M1	L-M3*
1	M	23	108	9	11	55	59	31	29	2	8	13	27	8	ND
2	M	9	71	17	17	42	49	23	20	1	2	11	28	3	2
3	M	30	102	10	11	19	17	7	15	0	3	6	14	3	0
4	M	19	91	5	7	24	35	10	15	0	5	5	9	2	0
5	M	17	26	15	15	46	47	22	23	0	13	3	19	0	1
6	F	23	41.5	11	8	ND	52	21	21	0	9	8	23	1	6
7	F	32	15	7	5	50	48	29	26	0	10	4	13	5	2
8	F	39	38	27	28	49	51	28	28	3	6	9	42	2	0
9	F	18	41	8	7	64	69	41	28	6	4	10	18	3	1
10	F	37	29	8	8	69	67	26	30	0	3	3	13	3	1
Average			56	11.7	11.7	46.4	49.4	23.8	23.5	1.2	6.3	7.2	20.6	3.0	1.4
Average for male donors			80	11.2	12.2	37.2	41.4	18.6	20.4	0.6	6.2	7.6	19.4	3.2	0.7
Average for female donors			33	12.2	11.2	58	57.4	29	26.6	1.8	6.4	6.8	21.8	2.8	2.0

ND, not done.

Determined by indirect immunofluorescence analysis using monoclonal antibodies and automated flow cytometry. Average negative background was: IgG_1, 5; IgG_2, 4; IgM, 6.

*L-M3 used direct immunofluorescence, background was 2.5.

Statistical analysis. For each specimen type, the percentage of lymphocytes positive for every cell marker was averaged and the SD was calculated. Student's *t* test was used to determine whether the average percentage obtained was significantly higher than the average negative background ($P < .05$). All percentages were then corrected by subtracting the background. By means of these corrected percentages, the mean and SD were again calculated. Student's *t* test was used to evaluate whether there was a significant difference between the percentage of positive lymphocytes obtained for a particular marker in one specimen type *v* another. A *P* value of $<.05$ was considered significant.

RESULTS

The percentages of lymphocytes in aspirates and peripheral blood was determined by performing a differential cell count on the aspirate and peripheral blood smears. These are presented in Tables 1 and 2. Cytospin preparations of the biopsy unicellular suspensions were of poor quality, and differential counts were not performed. The peripheral blood lymphocyte percentage for all donors was essentially normal (22% to 48% at this institution). The absolute number of lymphocytes in both aspirates and peripheral blood was calculated by multiplying the total WBC count by the lymphocyte percentage obtained from the differential cell count. Absolute numbers of T cells and B cells were then obtained by multiplying the absolute number of lymphocytes by the percentage positive for T cell and B cell markers. These values are given in Table 3 and indicate that there are far more T and B lymphocytes present in bone marrow than can be accounted for by peripheral blood contamination alone.

The results of the monoclonal antibody analysis for each specimen are also given in Tables 1, 2, and 4. All values given were corrected for background: for peripheral blood average, negative backgrounds were $IgG_1 = 4.0$; $IgG_2 = 3.2$; and

Table 3. Comparison of Absolute Numbers of Total Lymphocytes and T Lymphocytes in Aspirates and Peripheral Blood

		Aspirates			Peripheral Blood		
Donor	Sex	No. of Lymphs × $10^3/\mu L$	No. of B Cells × $10^3/\mu L$	No. of T Cells × $10^3/\mu L$	No. of Lymphs × $10^3/\mu L$	No. of B Cells × $10^3/\mu L$	No. of T Cells × $10^3/\mu L$
1	M	24.8	2.5	14.1	2.4	0.2	2.0
2	M	6.4	1.1	2.9	3.0	0.2	2.1
3	M	30.6	3.2	5.5	2.0	0.1	1.6
4	M	17.3	1.0	5.1	2.3	0.1	1.6
5	M	4.4	0.7	2.0	1.8	0.2	1.1
6	F	9.5	0.9	4.9	2.8	0.3	1.8
7	F	4.8	0.3	2.3	2.2	0.1	1.6
8	F	14.8	4.1	7.4	2.6	0.4	1.6
9	F	7.4	0.5	4.9	1.1	0.04	0.9
10	F	10.7	0.9	7.3	2.1	0.1	1.8
Average		13.1	1.5	5.6	2.2	0.2	1.6
SD		8.8	1.3	3.5	0.5	0.1	0.4

Absolute number of lymphocytes was calculated by multiplying the white blood cell count by the percentage of lymphocytes obtained from the differential count. Absolute number of T cells or B cells was calculated by multiplying the absolute number of lymphocytes by the average percentage of T cells or B cells. All of these values are given in Tables 1 and 2.

Table 4. Cell Marker Studies on Bone Marrow Biopsies From Ten Normal Donors: Cells (%) in Lymphocyte Window Positive for Specific Cell Surface Antigens

		B Cells		T Cells					Other			Myeloid	
		CD-19		CD-3	CD-5	CD-4	CD-8	CD-1	CD-16	CD-10		CD-15	
Donors	Sex	B-4	L-12	L-4	L-1	L-3	L-2	T-6	L-11	J-5	Ia	L-M1	L-M3*
1	M	5	6	11	20	10	17	0	6	0	9	6	ND
2	M	14	19	22	23	10	12	0	5	9	15	3	5
3	M	5	3	22	27	9	12	4	22	20	18	6	6
4	M	5	5	21	16	8	12	2	4	5	27	3	0
5	M	14	12	26	20	11	15	0	6	1	16	3	0
6	F	10	15	ND	22	9	18	11	5	3	23	0	7
7	F	8	6	20	10	9	15	3	13	4	16	11	3
8	F	14	14	31	32	6	22	0	18	10	27	3	0
9	F	3	4	27	25	12	15	4	9	10	25	6	0
10	F	2	0	24	ND	2	18	ND	ND	ND	ND	ND	ND
Average		8	8.4	22.7	21.7	8.6	15.6	2.7	9.8	6.9	19.5	4.5	2.6
Average for male donors		8.6	9.0	20.4	21.2	9.6	13.6	1.2	8.6	7.0	17.0	4.2	2.7
Average for female donors		7.4	7.8	25.5	22.2	7.6	17.6	4.5	11.2	6.8	22.7	5.0	2.5

ND, not done.

Determined by indirect immunofluorescence analysis using monoclonal antibodies and automated flow cytometry. Average negative background was: IgG_1, 8; IgG_2, 7; IgM, 6.

*L-M3 used direct immunofluorescence, background was 5.

IgM = 5.8. For aspirates, average negative backgrounds were $IgG_1 = 5.0$; $IgG_2 = 4.0$; and IgM = 6.0. For biopsies, average negative backgrounds were $IgG_1 = 8.0$; $IgG_2 = 7.0$; and IgM = 6.0. In essence, the peripheral blood analysis served as a control and ensured that the monoclonal antibodies were active and that donors had normal proportions of lymphocyte subsets. Aside from donor 8, who had somewhat elevated numbers of B cells, all donors had normal proportions of B cells and T cells in the peripheral blood. Donor results were also examined to determine whether there were any statistically significant differences ($P < .05$) in lymphocyte populations in males-females. No such differences were found.

T cell markers. The aspirates and biopsies showed significant differences in the percentages of lymphocytes marking with T cell markers (see Tables 2 and 4). The pan-T cell markers Leu-4 (CD-3) and Leu-1 (CD-5) showed 46% and 49% marking on aspirate lymphocytes, whereas percentages were only 23% and 22%, respectively, for biopsies. Similarly, Leu-3 (CD-4) and Leu-2 (CD-8) both showed 24% marking on aspirates, but only 9% and 16%, respectively, on biopsy lymphocytes. This difference was statistically significant for all four antibodies ($P < .0003$ for Leu-1; $P < .002$ for Leu-4; $P < .0005$ for Leu-3; and $P < .002$ for Leu-2). Generally the sum of Leu-3 and Leu-2 was close to the value obtained for the pan-T cell markers Leu-4 and Leu-1. T-6 (CD-1), a thymocyte marker, was not significantly different ($P > .05$) from background for either aspirates or biopsies (data not shown).

B cell markers. The pan-B cell markers B-4 and Leu-12 (CD-19) averaged 12% positivity in aspirates and 8% positivity in biopsies (Tables 2 and 4). There was no significant difference ($P > .05$) between the aspirate and biopsy values for B cell markers; neither were the aspirate or biopsy B cell markers significantly different from those of peripheral blood ($P > .05$).

J-5 (CD-10) equaled 7% in both aspirates and biopsies whereas Ia equaled 21% in aspirates and 20% in biopsies (Tables 2 and 4). There were no significant differences between biopsy and aspirate values for either of these early B cell markers ($P > .05$). When compared with those for peripheral blood, however, the values for J-5 and Ia in both aspirates and biopsies were significantly higher ($P < .003$ for J-5, aspirate *v* peripheral blood; $P < .05$ for J-5, biopsy *v* peripheral blood; $P < .007$ for Ia, aspirate *v* peripheral blood; and $P < .003$ for Ia, biopsy *v* peripheral blood).

NK cell markers. The percentage of cells positive for Leu-11 (CD-16) was 6% in aspirates and 10% in biopsies (Tables 2 and 4). This difference in values for Leu-11 was not statistically significant ($P > .05$), nor was either value for Leu-11 significantly different ($P > .05$) from the value of 4.5% obtained for peripheral blood (Table 1).

Myeloid markers. No significant staining for Leu-M3 was detected in either aspirates or biopsies in the lymphocyte population. Leu-M1 (CD-15) showed a small but definite positivity of 3% in aspirates and 5% in biopsies, indicating some contamination of the lymphocyte window with myeloid elements (Tables 2 and 4). When the myeloid elements were examined rather than the lymphocytes, the percentage of cells staining with Leu-M1 was >90% in biopsy specimens (data not shown).

Null cells. In aspirates, 30% of cells in the lymphocyte window did not stain with T, B, natural killer (NK), or myeloid markers. In biopsies, this value was 55%.

Two-color analysis. To determine the extent of myeloid cell contamination of the lymphocyte window, aliquots from three aspirate specimens were simultaneously labeled with two red fluorescing lymphoid markers (Leu-1 and Leu-12) and a green fluorescing myeloid marker (Leu-M1). These results were compared with the values obtained from the single-marker studies. There was very good agreement between the single-color and two-color analyses. In all three specimens, green fluorescence was virtually identical when the Leu-M1 value obtained from the single-marker study was compared with that obtained from the two-color analysis (data not shown). On average, <5% of cells in the lymphocyte window stained with Leu-M1. Because Leu-1 and Leu-12 should mark most lymphocytes, the percentage of cells exhibiting red fluorescence was taken to indicate the total percentage of lymphocytes in the lymphocyte window. There was <10% error when total lymphocytes were calculated from two-color or from one-color assays.

CD-4 to CD-8 ratios. Table 5 lists the ratios of CD-4 to CD-8 for each specimen type. Peripheral blood ratios of CD-4 to CD-8 were normal for all donors (1.3:1 to 2.3:1 at this institution). The bone marrow aspirates showed a significantly lower average ratio than did peripheral blood ($P < .02$). The biopsy ratio of CD-4 to CD-8 was significantly lower than both the peripheral blood ($P < .0005$) and the aspirate ratio of CD-4 to CD-8 ($P < .003$).

Table 5. Ratios of CD-4 to CD-8 Calculated for Specimens Taken From Ten Normal Donors

Donors	Peripheral Blood	Bone Marrow Aspirate	Bone Marrow Biopsy
Males			
1	1.2	1.1	0.6
2	1.1	1.1	0.8
3	1.6	0.5	0.8
4	1.6	0.7	0.7
5	1.1	1.0	0.7
Females			
6	1.2	1.0	0.5
7	1.8	1.1	0.6
8	1.5	1.0	0.3
9	3.2	1.5	0.8
10	1.3	0.9	0.1
Average for all patients	1.6	1.0	0.6
Average for male donors*	1.3	0.9	0.7
Average for female donors*	1.8	1.1	0.5

P-values: aspirate *v* peripheral blood $P < .02$; biopsy *v* peripheral blood $P < .0005$; biopsy *v* aspirate $P < .003$.

*There were no significant differences in ratios of CD-4 to CD-8 for males *v* females in any of the specimens.

DISCUSSION

Our results indicate that there are large numbers of mature, indigenous T cells in the bone marrow lymphocyte population, and that T cells of suppressor/cytotoxic phenotype predominate over those of helper/inducer phenotype. Bone marrow B cells, on the other hand, tend to be immature. Any assessment of lymphocyte subsets in bone marrow must be tempered by the fact that aspirate specimens are inevitably contaminated by peripheral blood. In two studies, peripheral blood contamination of aspirates was quantitated by the use of ^{51}Chromium labeled RBCs. These studies found 68% and 88% peripheral blood contamination by volume.[5,6] Examination of our aspirates revealed significant peripheral blood contamination in specimens 3, 7, 8, and 10 as evidenced: (a) by the high percentage of lymphocytes, (b) by a significant right shift in the myeloid series (data not shown), and (c) by decreased numbers of nucleated RBCs (data not shown). Three of the four contaminated aspirates came from females, and the average percentage of T cells was higher (although not significantly higher) for females than for males, suggesting that many T cells in marrow aspirates are actually contaminants.

We therefore assumed the worst possible case: that our aspirate specimens contained 100% of the WBCs present in an equal volume of peripheral blood. We then calculated the absolute numbers of total lymphocytes, B cells, and T cells present in the aspirate and peripheral blood. We also addressed the problem of peripheral blood contamination by analyzing both aspirates and biopsies. Biopsies have little peripheral blood contamination and, if there is no contamination in aspirates, the T cell percentages in biopsies and aspirates should be identical.

Using these two approaches, we found that our aspirates contained approximately three times as many T cells as an equal volume of peripheral blood (Table 3); therefore, peripheral blood could account for approximately one-third of the total aspirate T cell population in these specimens. When we examined the relative percentage of T cells in aspirates and biopsies we found, on average, 46% to 49% T cells in aspirates and 22% to 23% T cells in biopsies. Absolute counts were not available for biopsy lymphocytes; however, given the substantial difference in relative percentage of T cells between the aspirate and biopsy, one could assume that up to one-half of the aspirate T cells were due to peripheral blood contamination. These studies indicate, therefore, that there are large numbers of T cells in bone marrow, but that peripheral blood contamination of aspirates is substantial and increases the number of lymphocytes in the T cell subset by one-third to one-half.

The finding of substantial numbers of T cells in bone marrow agrees with the results of several other investigators, all of whom used the E rosette technique to identify T cells.[3,4,6,8] Working with rib fragments, rather than aspirates, DeGast and Platts-Mills reported a value of 27% for T cells.[4] Wulff and colleagues obtained a value of 38% in aspirates.[8] Both of these studies separated mononulcear cells from other WBCs by using a linear 5% to 15% sucrose gradient. Similarly, Gale and co-workers separated lymphocytes from aspirates using Ficoll-Hypaque, and found that 59% of the lymphocytes were E rosette positive.[6]

Other investigators have found lower values ranging from 0% to 12%.[1,2,5,7,9] Two of these investigators used linear 15% to 35% sucrose gradients to separate lymphocytes from aspirate specimens. The two studies showed 6% and 8% E rosette positivity.[1,5] Two other studies separated lymphocytes from marrow aspirates using a discontinuous Ficoll-Hypaque gradient in one case and a BSA gradient in the other, and found values of 9% and 0% T cells, respectively.[2,7] Finally, one group did not separate lymphocytes prior to E rosette analysis and found that 12% of aspirate lymphocytes were T cells; the rest were B cells and null cells.[9]

It is unlikely that the characteristics of the donor populations in the various studies explains the differences in T cell percentages obtained. De Gast and Platts-Mills suggested that their relatively high percentage of T cells was due to the advanced age of their patients (average age of 61 years), all of whom had underlying disease.[4] We obtained a similar percentage, however, in a normal population with an average age of 33 years. Furthermore, one investigator obtained a low percentage in a group of young normal volunteers similar to ours.[5]

The discrepancies may perhaps be explained by the different methods used for separation and staining. We found that sucrose gradients had very high osmolarities (data not shown) and led to substantial cell loss. Two investigators who obtained low percentages for T cells used sucrose gradients varying from 15% to 35%,[1,5] whereas higher values were obtained by investigators who used gradients varying from 5% to 15%.[4,8] However, the investigators who used discontinuous, isoosmotic Ficoll-Hypaque gradients obtained low numbers of T cells.[2]

The procedure which we used to process our aspirates has been found to cause no selective loss of lymphocyte subsets in peripheral blood when compared with the standard Ficoll-Hypaque procedure (Thomas Milson, personal communication, October 1985) and, by staining cells without first separating them, we ensured that there was no loss of cells during a separation procedure. Furthermore, we used monoclonal antibodies rather than the more cumbersome E rosette analysis to identify T cells. All our T cell markers were in good agreement with each other, suggesting that our results were accurate, and that we were indeed measuring appropriate T cell percentages.

We did note, however, that there was high nonspecific staining in the bone marrow biopsy specimens but not in the aspirates. This high background may have affected our results. Debris in the bone marrow biopsy specimens took up stain nonspecifically, causing substantial negative backgrounds. Despite this, well-defined cell populations were noted for T cell markers, especially Leu-4, suggesting that we were not being misled.

The ratios of CD-4 to CD-8 for the bone marrow specimens were quite low, being 0.6 for biopsies and 1.0 for aspirates. The peripheral blood ratio of CD-4 to CD-8 was normal, equaling 1.6. Because half of aspirate T cells could be peripheral blood contaminants, it is not suprising that the aspirate ratio of CD-4 to CD-8 was intermediate to those for peripheral blood and biopsies. Furthermore, in both aspirates and biopsies, the sum of CD-4 and CD-8 (which define mature T cells of helper and suppressor phenotype, respectively) equaled the total for CD-3 which defines total T cells, indicating that the indigenous marrow T cells were mature, as is also demonstrated by the fact that CD-1, which identifies thymocytes, was negative.

Previous experiments have shown that bone marrow lymphocytes rendered less help than peripheral blood lymphocytes in mixed lymphocyte cultures,[1,3] suggesting decreased numbers of helper/inducer cells. In 1981, Janossy and colleagues demonstrated high numbers of suppressor/cytotoxic cells in bone marrow using monoclonal antibodies and fluorescence microscopy.[11] Falcao and co-workers also reported a low ratio of CD-4 to CD-8 in aspirates from 25 normal donors.[12] The low helper-suppressor ratio in bone marrow suggests that the T cells in the marrow compartment may have a unique role in the marrow microenvironment.

Using the pan-B cell markers Leu-12 and B-4, which identify the CD-19 antigen, we found that the average percentage of B cells for aspirates and biopsies was 12% and 8%, respectively. With the exception of preplasma and plasma cells, CD-19 identifies all B lineage cells beyond the blast phase. Other studies of bone marrow have identified B cells by EA rosettes for Fc receptors, EAC rosettes for complement receptors, and by fluorescein-labeled anti-human antibodies. These studies found values for bone marrow B cells ranging from 7% to 34%.[1-3,5-9] Our percentages are not substantially different. Unlike T cells, the percentage of B cells was not significantly different in aspirates and biopsies. Small numbers of B cells are normally present in peripheral blood. Consequently, any blood contaminating an aspirate would not substantially change the B cell percentage.

The B cell marker Ia generally stained a higher percentage of cells than was CD-19 positive. Unlike CD-19, however, Ia marks a diverse population of cells (including B cells, activated T cells, stem cells, and some immature plasma cells), so this result is to be expected.

We found substantial numbers of cells that did not mark with any of our lymphocyte markers. These null cells constituted 55% of the lymphocyte population in biopsies and 30% in aspirates; other investigators using density gradient separations also found that between 41% and 78% of bone marrow lymphocytes were negative for lymphoid markers.[1-3,5,8,9] Some of these cells are nucleated red blood cells which contaminate the lymphocyte window (Thomas Milson, personal communication, October 1985).[11] Others may be Ia-positive stem cells and plasma cells. Several investigators found early lymphoid stem cells in their lymphocyte fraction.[2,7] It would be of interest to conduct further studies using TdT and PCA-1 to determine whether these null cells are in fact stem cells and plasma cells.

Approximately 7% of the lymphocyte population in both aspirates and biopsies was positive for CD-10, which defines CALLA positivity. Because only 8% of biopsy lymphocytes and 12% of aspirate lymphocytes marked as B cells, 7% CALLA positivity represents a high percentage of the total B cell population and suggests that most bone marrow B cells are immature. One other investigator found 7% CALLA positivity in the mononuclear cell fraction, but also found a much higher percentage of B cells.[13] Most investigators have found that CALLA-positive lymphocytes constitute ~0.1% to 3.0% of bone marrow lymphocytes.[14,15]

Our relatively high CALLA positivity may be due to the fact that other cells potentially present in bone marrow preparations (normal peripheral blood granulocytes, bone marrow stromal cells, and fibroblasts) are also CALLA positive.[14,16] Because we did not separate prior to staining, some of these cells may have been included in the lymphocyte window. Furthermore, in contrast to leukemic cells, the antigen density on CALLA-positive lymphocytes normally present in bone marrow is quite low.[17] Consequently, normal CALLA-positive cells are difficult to evaluate by flow cytometry because they are low-staining and barely detectable above background fluorescence. Thus, a finding of up to 10% CALLA-positive cells in bone marrow may represent contamination by nonlymphocytes that gate in the lymphocyte window rather than hematologic disease.

We found that 6% of aspirate lymphocytes marked for CD-16, which defines NK activity, and 10% of biopsy lymphocytes were CD-16 positive. Similarly, Talpaz and Spitzer found low NK activity in bone marrow aspirates from normal donors when they examined aspirates for spontaneous cell-mediated cytotoxicity.[18] Their study, however, did not attempt to quantitate numbers of NK lymphocytes.

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REFERENCES

1. Abdou NL, Alavi JB, Abdou NI: Human bone marrow lymphocytes: B and T cell precursors and subpopulations. Blood 47:423, 1976

2. Alley CD, MacDermott RP: Separation and characterization of human bone marrow mononuclear cells from aspirates and ribs. J Immunol Methods 32:223, 1980

3. DeGast GC, Platts-Mills TAE: Functional studies on lymphocytes in adult human bone marrow I. Immunoglobulin production *in vitro* after fractionation on a sucrose gradient and T/non-T cell separation. J Immunol 122:280, 1979

4. DeGast GC, Platts-Mills TAE: Functional studies on T cells in adult human bone marrow. Clin Exp Immunol 38:99, 1979

5. Fauci AS: Human bone marrow lymphocytes I. Distribution of lymphocyte subpopulations in the bone marrow of normal individuals. J Clin Invest 56:98, 1975

6. Gale RP, Opelz G, Kiuchi M, Golde DW: Thymus dependent lymphocytes in human bone marrow. J Clin Invest 56:1491, 1975

7. Geha RS, Gatien JG, Parkman R, Crain JD, Rosen FS, Merler E: Discontinuous density gradient analysis of human bone marrow: Presence of alloantigen-responsive, PHA-unresponsive cells in normal bone marrow; absence of B-lymphocytes in the bone marrow of patients with X-linked agammaglobulinemia. Clin Immunol Immunopathol 2:404, 1974

8. Wulff JC, Held H, Mueller-Hermelink HK, Schlaak, M: Purification and characterization of human bone marrow lymphocyte subpopulations: Evidence for the existence of an alloreactive immature T cell. Clin Exp Immunol 44:485, 1981

9. Borella L, Sen L: The distribution of lymphocytes with T and B cell surface markers in human bone marrow. J Immunol 112:836, 1974

10. Patrick CW, Swartz SJ, Harrison BS, Keller RH: Collection and preparation of hematopoietic cells for cell marker analysis. Lab Med 15:659, 1984

11. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein, G: Distribution of T lymphocyte subsets in the human bone marrow and thymus: An analysis with monoclonal antibodies. J Immunol 126:1608, 1981

12. Falcao RP, Voltarelli JC, Bottura C: T lymphocyte subpopulations in the peripheral blood and bone marrow of patients with aplastic anemia. Blut 50:103, 1985

13. Hokland P, Nadler LM, Griffin, JD, Schlossman, SF, Ritz J: Purification of common acute lymphoblastic leukemia antigen-positive cells from normal human bone marrow. Blood 64:662, 1984

14. Greaves MF, Hariri G, Newman RA, Sutherland DR, Ritter MA, Ritz J: Selective expression of the common acute lymphoblastic leukemia (gp 100) antigen on immature lymphoid cells and their malignant counterparts. Blood 61:628, 1983

15. Greaves M, Janossy G: Patterns of gene expression and the cellular origins of human leukaemias. Biochim Biophys Acta 516:193, 1978

16. Cossman J, Neckers LM, Leonard WJ, Greene WC: Polymorphonuclear neutrophils express the common acute lymphoblastic leukemia antigen. J Exp Med 157:1064, 1983

17. Greaves M, Delia D, Janossy G, Rapson N, Chessells J, Woods M, Prentice G: Acute lymphoblastic leukaemia associated antigen IV. Expression on non-leukaemic 'lymphoid' cells. Leuk Res 4:15, 1980

18. Talpaz M, Spitzer G: Low natural killer cell activity in the bone marrow of healthy donors with normal killer cell activity in the peripheral blood. Exp Hematol 12:629, 1984

The Regulatory Role of Interleukin 2–Responsive T Lymphocytes on Early and Mature Erythroid Progenitor Proliferation

By Zeev Estrov, Chaim Roifman, Yao-Ping Wang, Tom Grunberger, Erwin W. Gelfand, and Melvin H. Freedman

To analyze the role of T lymphocytes in human erythropoiesis, we evaluated the effect of recombinant interleukin 2 (IL 2) on marrow CFU-E and BFU-E colony formation in vitro. IL 2 resulted in an increase in CFU-E and BFU-E colony numbers in a dose-dependent manner. This increase could be prevented by anti-Tac, a monoclonal antibody to the IL 2 receptor. Moreover, anti-Tac on its own resulted in an overall decrease in colony numbers. Depletion of marrow adherent cells did not alter the effect of either IL 2 or anti-Tac on colony growth. Following the removal of marrow T lymphocytes, CFU-E and BFU-E colony formation proceeded normally; however, the effects of IL 2 and anti-Tac were markedly diminished. Readdition of T lymphocytes to the cultures restored the IL 2 effect. Although T lymphocytes were not themselves essential for in vitro erythropoiesis, our studies suggest that IL 2 and IL 2–responsive T cells can regulate both early and mature stages of erythroid differentiation.

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SINCE the development of in vitro erythroid colony assays, many conflicting observations regarding the influence of T cells on erythroid burst-forming units (BFU-E) have been reported. Some investigators[1,2] found that monocytes rather than T lymphocytes are the major population of cells that produce burst-promoting activity (BPA), whereas others have shown that T lymphocytes are a potent source of BPA[3-7] and have a regulatory effect on BFU-E.[8,9] However, the influence of T cells on erythroid colony-forming units (CFU-E) has not been studied in depth, and it is accepted that the terminal stages of erythroid differentiation are regulated by erythropoietin.[7] Interleukin 2 (IL 2), which is produced by T lymphocytes[10] and is required for the proliferation of activated T cells, was found to enhance T lymphocyte production of a variety of colony-stimulating factors.[11]

In the present study, we show the influence of IL 2 and of an antibody that blocks its T cell receptor sites[12] on marrow erythroid colony growth in vitro. Using CFU-E and BFU-E culture assays, we demonstrate that T lymphocytes responsive to IL 2 regulate early (as expressed by BFU-E) as well as differentiated (as expressed by CFU-E) erythroid proliferation in vitro, indicating their regulatory role in erythropoiesis.

MATERIALS AND METHODS

Preparation of cells. Heparinized (10 U/mL) bone marrow (BM) was obtained from hematologically normal adult volunteers. The donors were advised of the procedure and attendant risks and gave informed consent. These studies were approved by the Human Experimentation Committee of our institution. The BM cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centifuged (200 g, 10 °C) for 20 minutes to remove neutrophils and red cells.

Adherent cell removal. The mononuclear cells were depleted of adherent cells by incubation on plastic Petri dishes with 10% fetal calf serum (FCS) for 90 minutes at 37 °C. In this modification of the technique described by Shaw et al,[13] nonadherent cells were removed by gentle pipetting and repeated vigorous washing of the plates with medium. This procedure was repeated until no further cells adhered to the plastic Petri dishes. The nonadherent cell fraction contained <3% monocytes according to an analysis of cytocentrifuge smears counting 300 to 500 cells using Wright's stain, nonspecific (alpha naphthyl butyrate) esterase stain,[14] and a modification of an immunocytochemical technique[15] with anti–MY-4 monoclonal antibody (Coulter Immunology, Hialeah, Fla) to identify monocyte-promonocyte cells on nonadherent cell smears.

Double T cell depletion. Post-Ficoll BM mononuclear cells were incubated with neuraminidase-sensitized sheep erythrocytes (SRBC) at 4 °C for two hours, followed by gradient centrifugation. This procedure was repeated twice. Cells at the interface contained <0.5% T cells as assessed by AET-sensitized SRBC rosette formation[16] and using anti–Leu-4 and anti–Leu-5 monoclonal anti–T cell antibodies (Becton Dickinson, Mountain View, Calif). T lymphocytes were obtained by recovery of rosetted cells from the pellet following lysis with Tris-buffered ammonium chloride. In the reconstitution experiments, these T lymphocytes were washed and counted, and either 5%, 25%, or 100% of the cells were added back to 1×10^5 T cell–depleted BM cells.

Lymphocyte removal using CAMPATH-1. BM cells obtained following Ficoll-Paque gradient centrifugation were then washed once in phosphate-buffered saline (PBS) and treated with a monoclonal rat antihuman lymphocyte antibody (CAMPATH-1), as previously described.[17,18] No detectable T lymphocytes following this treatment were found by E rosette formation or by indirect immunofluorescence with anti–Leu-1 (Becton Dickinson, Mountain View, Calif).

Interleukin 2. IL 2, derived from cDNA for the human IL 2 gene expressed in *Escherichia coli* (recombinant IL 2),[10,11] was provided by Biogen Research Corp (Cambridge, Mass). Following reconstitution in α-medium, the recombinant IL 2 was assayed on a murine IL 2–dependent cell line to confirm its activity[19] and was compared to IL 2 obtained from the Gibbon cell line MLA 144, which showed similar effects. Recombinant IL 2, whose stimulatory activity was blocked by anti–IL 2 antibodies,[20] was added to cultures at concentrations of 1.0, 2.5, 5, and 10 U/mL.

Anti-Tac antibody.[12] Monoclonal anti-Tac antibody directed against the IL 2 receptor was provided by W. Greene (National Institutes of Health, Bethesda, Md). Its effect on an IL 2–dependent T cell line was reversible after removal of the antibody and could be overcome by high concentrations of IL 2. Hybridoma supernatant tested as a control for anti-Tac was inactive in this system as well as

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in the erythroid culture assay. Anti-Tac was added at the initiation of culture in four different dilutions of ascites fluid ranging from 1:500 to 1:5,000. For control, anti–Leu-1 (Becton-Dickinson, Sunnyvale, Calif), a monoclonal pan-T antibody of identical subclass as anti-Tac (IgG_{2a}) was added to cultures in final concentrations of 0.025 to 0.5 μg/mL.

Erythroid colony culture assay. Post-Ficoll nucleated BM cells (1×10^5) were cultured in 0.8% methylcellulose with α-medium, 30% FCS, and 1% bovine serum albumin at a final volume of 1 mL per culture plate.[21] An IL 2–free (tested on a T cell line) human erythropoietin, 1.0 U/mL (British Columbia Cancer Research Institute, Vancouver, BC), was added to stimulate erythroid colony growth. Cultures were set up in duplicate or quadruplicate and maintained at 37 °C in 5% CO_2 in a humidified atmosphere. Colonies were counted in the culture plates using an inverted microscope. Culture plates were observed daily starting from day 4 of incubation. A cluster of eight or more cells showing first signs of hemoglobulinization, which occurred at day 7, was defined as a CFU-E colony, and an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies counted at day 14 was defined as a BFU-E colony.[22]

Statistical analysis. The probability of significant differences between colony numbers was determined by Student's *t* test.

RESULTS

Effects of the addition of IL 2 and anti-Tac on the growth of CFU-E and BFU-E. Figures 1 and 2 illustrate the results of five separate experiments from different donors in which cells were cultured in the presence or absence of either IL 2 or anti-Tac. The stimulatory effect of IL 2 on CFU-E and BFU-E and the inhibitory effect of anti-Tac were evident in a dose-dependent manner in all experiments. The maximum colony increase comparing cultures without IL 2 and those with 10 U/mL IL 2 was 38 CFU-E and 10 BFU-E/10^5 cells plated. However, the IL 2–induced increments on CFU-E and BFU-E colony numbers with 10 U/mL and the inhibitory effects of anti-Tac with a 1:500 dilution were both significant ($P < .03$ and $P < .05$, respectively). The addition of 10 U/mL IL 2 and a 1:500 dilution of anti-Tac simultaneously to cultures did not produce a significant change compared with the control (109 ± 6 *v* 103 ± 4 CFU-E and 36 ± 3 *v* 34 ± 2 BFU-E), suggesting a negation of their respective effects and ruling out a nonspecific toxic effect of anti-Tac. In the control cultures, anti–Leu-1, the monoclonal pan-T antibody of identical subclass as anti-Tac, had no effect on CFU-E and BFU-E colony growth.

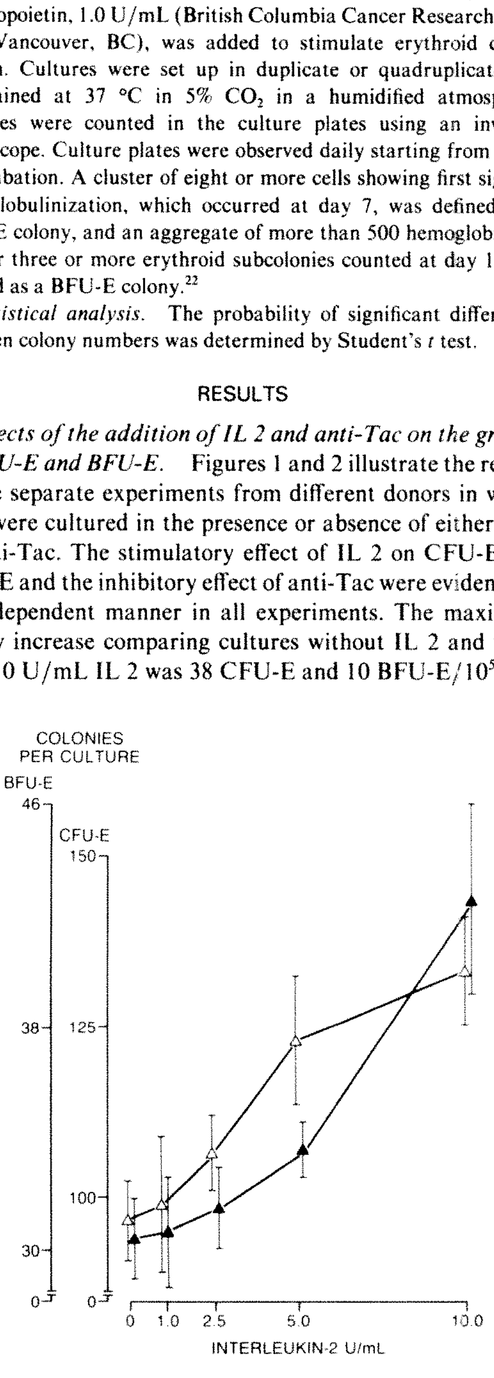

Fig 1. **The number (mean ± SD of duplicate dishes) of marrow CFU-E and BFU-E (colonies/10^5 cells plated) obtained from five separate BM samples in the presence of various concentrations of IL 2. CFU-E = △—△; BFU-E = ▲—▲.**

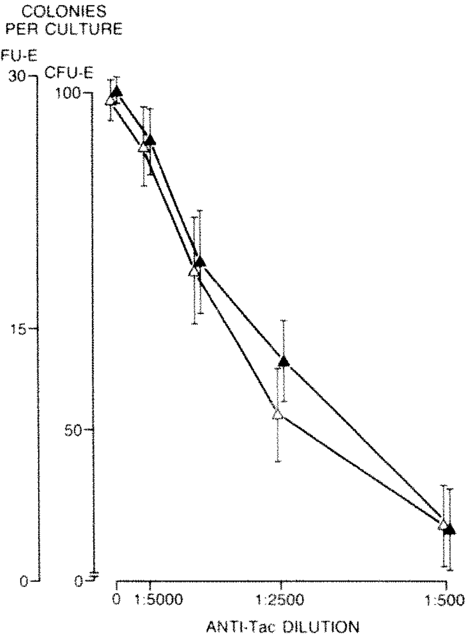

Fig 2. **The number (mean ± SD of duplicate dishes) of marrow CFU-E and BFU-E (colonies/10^5 cells plated) obtained from five separate BM samples in the presence of four different dilutions of ascites fluid containing anti-Tac. CFU-E = △—△; BFU-E = ▲—▲.**

Effects of IL 2 and anti-Tac on lymphocyte-depleted BM (using CAMPATH-1) in the presence and absence of adherent cells. In Fig 3, the combined results of two separate experiments using BM from donors not used in Figs 1 and 2 are presented. The removal of adherent cells (column 3 of the Fig) did not alter the effect of IL 2 or anti-Tac on colony growth. The stimulatory effects of IL 2 on CFU-E and BFU-E colony numbers and the inhibitory effects of anti-Tac were both significant ($P < .05$) and were more evident than the results of the initial experiments shown in Figs 1 and 2. The removal of T lymphocytes from marrow cells by CAMPATH-1 negated the effects of IL 2 and anti-Tac in the presence or absence of adherent cells (columns 2 and 4 of the Fig).

Effects of IL 2 and anti-Tac on cultures of T lymphocyte–depleted BM and the addition of autologous T lymphocytes. Using marrow from which T lymphocytes were depleted by double E rosette depletion, neither the addition of IL 2 nor anti-Tac had any effect on colony numbers in the

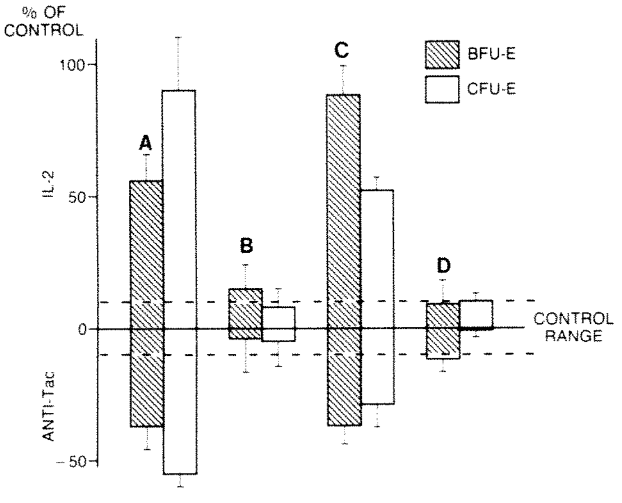

Fig 3. The effect of IL 2 and anti-Tac on four different marrow cell preparations: (A) post-Ficoll; (B) lymphocyte depleted; (C) adherent cell depleted; and (D) lymphocyte and adherent cell depleted. The results (mean ± SD of quadruplicate dishes) are expressed as a percentage of the increase or decrease in control colony numbers, which was defined as the result obtained in the absence of IL 2 and anti-Tac.

CFU-E or BFU-E culture assay (columns 1 and 2 of Table 1). No change in colony numbers was observed on adding back 5% of the removed T lymphocytes to the cultures (data not presented). However, on adding 25% of the removed T cells to 1 × 10^5 T-depleted BM cells, IL 2 produced a colony increase of 28% CFU-E ($P < .02$) and 50% BFU-E ($P < .03$); anti-Tac resulted in a 24% reduction of CFU-E ($P < .02$) and 18% BFU-E ($P < .03$). Similar results were demonstrated on adding back 100% of the removed T cells. As a control, T cells were plated for erythroid colony growth, and no BFU-E or CFU-E colonies were identified with or without IL 2 or anti-Tac.

DISCUSSION

The data reported in the present study clarify some aspects of the regulatory role of T cells on the control of erythropoiesis. We examined the effect of marrow T cells on erythroid colony growth from eight marrows. The studies were performed on post-Ficoll mononuclear cell suspensions as well as on cell preparations following adherent cell removal, a treatment with a monoclonal antilymphocyte antibody (CAMPATH-1) that depleted B as well as T cells,[17] and after double E rosette depletion. Both methods for T cell depletion were highly efficient and comparable. IL 2 was used in the study as an enhancer of T lymphocyte function,[11] whereas the monoclonal antibody, anti-Tac, was used to block the effect of IL 2.[12,23] The effect of anti-Tac was reversible and could be overcome by high concentrations of IL 2; thus the possibility of an immune complex or a toxic nonspecific inhibitory effect could be excluded.

Table 1. The Effect of IL 2 and Anti-Tac on Marrow CFU-E and BFU-E Colony Growth

	T-Depleted Marrow		T Cell Reconstituted Marrow	
	CFU-E	BFU-E	CFU-E	BFU-E
Control	175 ± 5	57 ± 2	235 ± 12	51 ± 3
IL 2 (10 U/mL)	177 ± 6	57 ± 1	300 ± 12	77 ± 6
Anti-Tac (dilution of 1:500)	176 ± 10	58 ± 2	177 ± 6	42 ± 1

T cells were removed by E rosette depletion, and then 25% of the T lymphocytes were added back to 1 × 10^5 T cell-depleted BM cells. (Values shown are the mean ± SD of duplicate IL 2- and anti-Tac-treated BM samples and quadruplicate control cultures.)

The major findings were that IL 2 enhanced CFU-E and BFU-E colony growth whereas anti-Tac inhibited it in a dose-dependent manner. The anti-Tac or IL 2 effects were not observed when T cells were removed from the BM cell suspension following treatment with CAMPATH-1 or after double E rosette depletion. Enhancement by IL 2 or inhibition by anti-Tac were observed only when at least 25% of the original T cell population was added back to E rosette (T cell) depleted BM cells. Thus, it is unlikely that the IL 2 and anti-Tac effect was a direct one on erythroid progenitors but was likely mediated through T cells. The adherent cell fraction played a minor role since their removal did not alter the findings.

Although Zuckerman reported that human BFU-E growth in culture was dependent on the presence of monocytes but not T lymphocytes,[1] our findings that adherent cells are not essential for erythropoiesis are in agreement with other laboratories.[3,4] Moreover, we have convincingly confirmed that CFU-E growth in culture, although not dependent on T cells, is enhanced by T cells, as originally suggested by Mangan et al.[24]

An analysis of the published studies dealing with nonhematopoietic cellular regulation of erythropoiesis reveals several technologic problems that could account for the contradictory reports that have emerged. First, marrow cell populations are highly heterogenous with varying numbers of subpopulations from sample to sample, even after Ficoll or other cell separation procedures. The interaction of the subpopulations cannot be quantitated or controlled and could make a comparison of various studies difficult and confusing. Second, the methods for removal of specific cell fractions are imperfect and do not always yield a complete depletion of the cell population of interest. A small number of residual cells could influence erythroid colony growth in vitro and lead to erroneous conclusions. In addition, the depletion techniques may not remove progenitors that in turn could differentiate in culture and ultimately affect erythroid colony growth as previously suggested[1,25,26] or secrete interleukin 1 (a macrophage product that is probably necessary for IL 2 production).[27] With respect to the T cell depletion techniques that we used, detailed studies by others using various techniques[17,28,29] to detect residual T cells after rosetting or CAMPATH-1 cytolysis have shown that no completely effective method is available. Similarly, with the adherence method to remove monocytes and macrophages we expect some residual cells in the depleted fraction. Another factor that must be taken into account in the interpretation of data is the enrichment effect on CFU- and BFU-E when T cells and/or adherent cells are removed, but when the same number of cells are plated in all the studies.

In our experiments, we attempted to circumvent many of these problems by using recombinant IL 2, a specific stimulator of activated T cells,[10,11] and the monoclonal anti-Tac antibody,[12] a blocker of IL 2 receptor sites. These two reagents afforded an opportunity to explore the interaction of erythroid progenitors and T cells in a functional way. Another novel approach was to deplete T cells using the monoclonal antibody CAMPATH-1[17] in addition to the standard E rosette depletion technique. The T cell depletion studies confirmed that CFU-E and BFU-E growth was T cell independent, and the CAMPATH-1 studies indicated that CFU-E and BFU-E growth was probably B cell independent as well since the antibody eliminates both T and B cells.[17]

According to previous studies, T lymphocytes can regulate BFU-E growth[3-9] primarily by producing BPA.[3-7] Our findings indicate that the erythropoietic regulatory role of the T lymphocytes can be mediated by IL 2 and affect CFU-E as well as BFU-E. We have concluded that T lymphocytes are not essential for in vitro erythropoiesis[1,7]; however, T cells do have a regulatory role in early and mature erythroid progenitor proliferation in vitro.

REFERENCES

1. Zuckerman KS: Human erythroid burst-forming units growth in vitro is dependent on monocytes but not T-lymphocytes. J Clin Invest 67:702, 1981

2. Nomdedeu B, Gormus BJ, Banisadre M, Rinehart JJ, Kaplan ME, Zanjani ED: Human peripheral blood burst forming unit (BFU-E): Evidence against T-lymphocyte requirement for proliferation in vitro. Exp Hematol 8:845, 1980

3. Nathan DG, Chess L, Hilman DG, Clark B, Bernard J, Merler E, Houseman D: Human erythroid burst forming unit: T-cell requirement for proliferation in vitro. J Exp Med 147:324, 1978

4. Mangan KF, Chikkappa G, Bieler LZ, Scharfman WB, Parkinson DR: Regulation of human blood erythroid burst forming unit (BFU-E) proliferation by T-lymphocyte subpopulations defined by Fc receptors and monoclonal antibodies. Blood 59:990, 1982

5. Torok-Storb B, Hansen JA: Modulation of in vitro BFU-E growth by normal Ia positive T cells is restricted by HLA-DR. Nature 298:473, 1982

6. Golde DW, Bersch N, Quan SG, Lusis AJ: Production of erythroid-potentiating activity by a human T-lymphoblast cell line. Proc Natl Acad Sci USA 77:593, 1980

7. Linch DC, Nathan DG: T cell and monocyte derived burst promoting activity directly act on erythroid progenitor cells. Nature 312:775, 1984

8. Wisniewski D, Strife A, Wachter M, Clarkson B: Regulation of human peripheral blood erythroid burst forming unit growth by T-lymphocytes and T-lymphocyte subpopulations defined by OKT_4 and OKT_8 monoclonal antibodies. Blood 65:456, 1985

9. Pistoia V, Ghio R, Nocera A, Leprini A, Perata A, Ferrarini M: Large granular lymphocytes have a promoting activity on human peripheral blood erythroid burst forming units. Blood 65:464, 1985

10. Morgan DA, Ruscetti FW, Gallo RC: Selective in vitro growth of T-lymphocytes from normal human bone marrows. Science 193:1007, 1976

11. Greenberger JS, Krensky AM, Messner H, Burakoff SJ, Wandl U, Sakakeeny MA: Production of colony stimulating factor(s) for granulocyte-macrophage and multipotential (granulocyte/erythroid/megakaryocyte/macrophage) hematopoietic progenitor cells (CFU-GEMM) by clonal lines of human IL-2 dependent T-lymphocytes. Exp Hematol 12:720, 1984

12. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. J Immunol 126:1393, 1981

13. Shaw GM, Levy PC, LoBuglio AF: Human monocyte antibody–dependent cell mediated cytotoxicity to tumor cells. J Clin Invest 62:1172, 1978

14. Tucker SB, Pierre RV, Jordan RE: Rapid identification of monocytes in a mixed mononuclear cell preparation. J Immunol Methods 14:267, 1977

15. Li CY, Ziesmer SC, Yam LT, English MC, Janckila AJ: Practical immunochemical identification of human blood cells. Am J Clin Pathol 81:204, 1984

16. Boyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21:1, 1968

17. Hale G, Bright S, Chumbley G, Hoang T, Metcalf D, Munro AJ, Waldmann H: Removal of T cells from bone marrow for transplantation: A monoclonal antilymphocyte antibody that fixes human complement. Blood 62:873, 1983

18. Waldmann H, Hale G, Cividalli G, Weshler Z, Manor D, Rachmilewitz EA, Polliak A, Or R, Weiss L, Samuel S, Brautbar C, Slavin S: Elimination of graft versus host disease by in vitro depletion of alloreactive lymphocytes with a monoclonal rat anti-human lymphocyte antibody (CAMPATH-1). Lancet 2:483, 1984

19. Gillis S, Ferm MM, Ou W, Smith KA: T-cell growth factor: Parameters of production and a quantitative microassay for activity. J Immunol 120:2027, 1978

20. Smith KA, Favata MF, Oroszlan S: Production and characterization of monoclonal antibodies to human interleukin 2: Strategy and tactics. J Immunol 131:1808, 1983

21. Iscove NN, Sieber F, Winterhalter KH: Erythroid colony formation in cultures of mouse and human marrow. Analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose concanavalin A. J Cell Physiol 83:309, 1974

22. Gregory CJ, Eaves AC: Human marrow cells capable of erythropoietic differentiation in vitro: Definition of three erythroid colony responses. Blood 49:855, 1977

23. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC: A monoclonal antibody that appears to recognize the receptor of human T-cell growth factor; partial characterization of the receptor. Nature 300:267, 1982

24. Mangan KF, Chikkappa G, Farley PC: T gamma ($T\gamma$) cells suppress growth of erythroid colony forming units in vitro in the pure red cell aplasia of B-cell chronic lymphocytic leukemia. J Clin Invest 70:1148, 1982

25. Reid CDL, Baptista LC, Chanarin I: Erythroid colony growth in vitro from human peripheral blood null cells: Evidence for regulation by T-lymphocytes and monocytes. Br J Haematol 48:155, 1981

26. Verma DS, Spizer G, Beran M: DR antigen positive monocyte-macrophages control granulocyte-macrophage colony stimulating activity and burst promoting activity elaboration in man. Am J Hematol 12:403, 1982

27. Gillis S: Interleukin 2: Biology and biochemistry. J Clin Immunol 3:12, 1983

28. Martin PJ, Hansen JA: Quantitative assays for detection of residual T cells of T-depleted human marrow. Blood 65:1134, 1985

29. Levitt L, Kipps TJ, Engleman EG, Greenberg PL: Human bone marrow and peripheral blood T lymphocyte depletion: Efficacy and effects of both T cells and monocytes on growth of hematopoietic progenitors. Blood 65:663, 1985

Chromosomes With One, Two, Three, and Four Fetal Globin Genes: Molecular and Hematologic Analysis

By A.V.S. Hill, D.K. Bowden, D.J. Weatherall, and J.B. Clegg

Analysis of DNA from 852 Island Melanesians has revealed a high frequency of single- and triple-γ-globin genes in this population. Homozygotes for triple- and single-γ genes have normal hematologic findings, normal hemoglobin F (HbF) levels, and when there is coexisting α thalassemia, appropriate levels of Bart's Hb (γ_4) at birth. In addition, we have identified an individual with a quadruple-γ gene chromosome who also has a normal HbF level. All single-γ genes were ${}^{A}\gamma$, all triple-γ genes ${}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$, and the quadruple-$\gamma$ gene ${}^{G}\gamma{}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$. Analysis of ${}^{G}\gamma$:${}^{A}\gamma$ ratios in cord bloods and HbF levels in adults showed that these additional γ genes are expressed and are down regulated appropriately by the fetal to adult Hb switch. Analysis of the restriction enzyme haplotypes of these various chromosomes indicates that intrachromosomal cross-overs are more likely to have produced these variants than interchromosomal recombination events.

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MOLECULAR ANALYSIS of mutant globin genes has provided new insights into mechanisms of gene expression and its control in man.[1] Broadly, these mutations may be divided into single-base changes and larger rearrangements,[2] including gene deletions, duplications, and one case of an inversion.[3] The phenotypic results of point mutations correlate well with the effects of such changes on transcription, RNA processing, and translation. However, despite the analysis of over 20 different larger rearrangements in the β-globin complex, it has not been possible to develop a unifying model to account for their phenotypic effects. The major clinical interest in these β-globin locus rearrangements derives from the insight they may provide into the processes controlling the developmental switch from fetal to adult hemoglobin (Hb) production.

Most of the deletions described previously in the β-globin complex appear to have been produced by recombination between apparently unrelated sequences, so-called illegitimate recombination events.[4] These may be contrasted with homologous recombination events in which deletions and duplications are produced by unequal but homologous cross-overs. Although rearrangements produced by homologous recombination have been studied in detail, particularly in the α-globin complex,[5] the mechanism of such cross-overs—whether interchromosomal or intrachromosomal—as well as their frequency remain unknown.

During a study of the globin genes of Melanesians from the archipelago of Vanuatu in the Southwest Pacific[6] we found a remarkably high frequency of triple–[7] and single–γ-globin[8] gene chromosomes as well as the normal two-gene (${}^{G}\gamma{}^{A}\gamma$) chromosome. In addition, we discovered one family with a quadruple (${}^{G}\gamma{}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$)-$\gamma$ gene chromosome,[6,9] possibly derived from an unequal cross-over between two $\gamma\gamma\gamma$ gene sister chromatids. Heterozygotes for single- and triple-γ genes have been reported previously, but they are rare in most populations and their phenotypes have been incompletely defined. By analysis of ${}^{G}\gamma$:${}^{A}\gamma$ ratios in cord bloods and HbF levels in adults heterozygous and homozygous for these rearrangements we find that neither a deletion of 5 kilobases (kb) nor an insertion of 5 or 10 kb of DNA around the fetal γ genes significantly alters the pattern of Hb switching; adults with from two to six γ-globin genes have normal HbF levels. Furthermore, analysis of linked restriction enzyme polymorphisms on chromosomes with one, two, three, and four γ genes gives some insight into the mechanism of recombination that produces these rearrangements.

MATERIALS AND METHODS

Adult samples were collected from healthy individuals on numerous islands throughout the archipelago of Vanuatu (formerly the New Hebrides). Cord bloods were collected at the Central Hospital, Port Vila, Vanuatu. Thirty samples from Melanesians resident on the large southerly island of New Caledonia were supplied by Dr S.W. Serjeantson. Samples were kept refrigerated until blood counts were performed within five days of collection. Blood counts and hemoglobin electrophoresis and quantitation were performed by standard methods.[10] ${}^{G}\gamma$:${}^{A}\gamma$ ratios on cord bloods, DNA extraction, digestion, electrophoresis, and Southern blotting were performed as described previously.[10,11] The probes used were (1) a 3.2-kb Hind III fragment including the entire ${}^{A}\gamma$ gene; (2) a 1.7-kb Bgl II/Xba I fragment containing the $\psi\beta$ gene; (3) pϵ, a recombinant plasmid with a 1.3-kb Bam H1/Eco R1 ϵ fragment subcloned into pBR322; (4) a 1.5-kb Pst I fragment containing the entire α1-globin gene; (5) a 2.8-kb EcoR1/Bam H1 fragment containing the entire ζ2 gene; (6) a 4.5-kb Pst I fragment containing the entire β-globin gene; and (7) pRK 29,[12] a plasmid that contains the 1.2-kb Eco R1 fragment 18 kb 3′ to the β gene.

RESULTS

Triplicated and single γ genes. The human β-like globin gene complex consists of five genes and a pseudogene arranged in the order 5′-ϵ-${}^{G}\gamma$-${}^{A}\gamma$-$\psi\beta$-δ-β-3′ (Fig 1). The fetal globin genes ${}^{G}\gamma$ and ${}^{A}\gamma$ are contained within a 13-kb Bgl II fragment. Triplicated γ genes produce a new 18-kb fragment and single-γ genes a new 8-kb fragment (Fig 2) on hybridization with a γ gene probe. Using this screening strategy, we analyzed 852 Melanesians from the archipelago of Vanuatu and the island of New Caledonia and found gene frequencies of 3.9% for triplicated γ genes ($\gamma\gamma\gamma$) and 6.5% for single γ genes ($-\gamma$) (Table 1). Interestingly the frequency of these variants varies from island to island. In particular, the two

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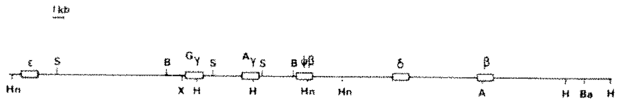

Fig 1. Restriction enzyme map of the β-globin gene cluster. Polymorphic restriction enzyme sites are shown below and the Bg1 II and Sph I sites used to determine γ-globin gene copy number above the complex. The Hind III site (H) at the 3′ end of the complex is a new restriction enzyme site polymorphism found in Melanesians and Polynesians. Key: B, Bgl II; S, Sph I; Hn, Hinc II; H, Hind III; X, XmnI; A, Ava II; Ba, Bam H1.

ethnically Polynesian groups sampled from the islands of Futuna and Emae[13] both had very high frequencies of single-γ genes. The frequencies of these $\gamma\gamma\gamma$ and $-\gamma$ chromosomes is much higher than in any other population studied to date (ref 14, and our unpublished observations).

A new quadruple γ gene. As well as the 18-kb ($\gamma\gamma\gamma$), 13-kb ($\gamma\gamma$), and 8-kb ($-\gamma$) kb bands previously described, one individual had a 23-kb band as well as a normal 13-kb band on Bgl II digestion (Fig 2). This variant was analyzed further using the enzymes Eco RV and Bcl I. Normal ($\gamma\gamma$) chromosomes give 15- and 18-kb bands, respectively, with these enzymes, which also cut outside the γ genes. A homozygote for the triple γ gene gave bands 5 kb larger in each case, and the individual with the ~23-kb band on Bgl II digestion had EcoRV and Bcl I γ bands 10 kb larger than normal. It seemed likely, therefore, that this individual had a quadruple ($\gamma\gamma\gamma\gamma$)-gene chromosome as well as a normal ($\gamma\gamma$) chromosome. However, to rule out the possibility that the larger band was due to an insert of foreign DNA near the γ genes, further digests with Hind III, Eco R1, and Sac I were performed, and, consistent with a $\gamma\gamma\gamma\gamma$ arrangement, only normal bands were found. Sph I gives a new 5-kb band in the presence of a triplicated γ-globin gene chromosome. Inspection of the Sph I map (Fig 1)[15] shows that, with a quadruple-γ gene produced by a homologous cross-over, no additional band should be detectable but the 5-kb band should be of double intensity. This was the case as shown in Fig 3.

The sample found to have a quadruple-γ gene was one of the collection of cord bloods. Follow-up of the family of this individual showed she had inherited the $\gamma\gamma\gamma\gamma$ chromosome from her father who gave identical results on DNA mapping (see the following).

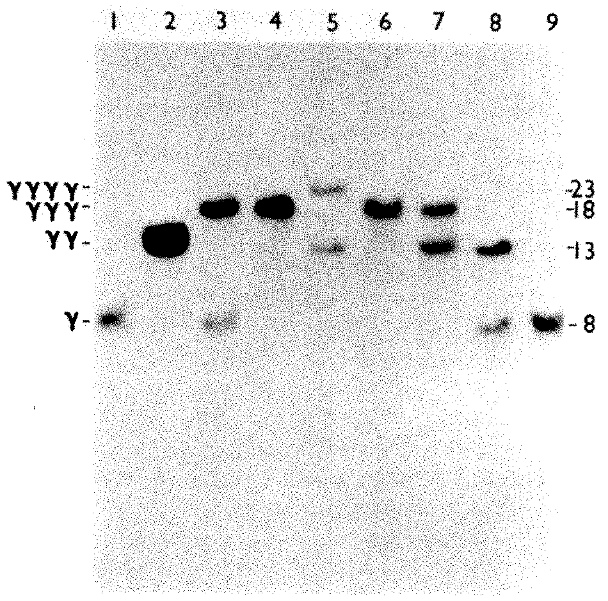

Fig 2. Restriction fragments seen in nine individuals with various γ genotypes following Bg1 II digestion and hybridization with the γ probe. The individuals have the following genotypes: lanes 1 and 9, $-\gamma/-\gamma$; lane 2, $\gamma\gamma/\gamma\gamma$; lane 3, $\gamma\gamma\gamma/-\gamma$; lanes 4 and 6, $\gamma\gamma\gamma/\gamma\gamma\gamma$; lane 5, $\gamma\gamma\gamma\gamma/\gamma\gamma$; lane 7, $\gamma\gamma\gamma/\gamma\gamma$; lane 8, $-\gamma/\gamma\gamma$. Band sizes are indicated in kilobases.

All chromosomes have one $^{A}\gamma$ gene. The amino acid sequences of the $^{G}\gamma$- and $^{A}\gamma$-globins differ at residue 136, the result of a single-base change at this position between the $^{G}\gamma$ and $^{A}\gamma$ genes.[16] This difference can be detected using the enzyme Pst I, and thus it was shown that the previously reported single-γ gene[8] was $^{A}\gamma$ and the triple-γ gene arrangement found in Vanuatu and elsewhere, $^{G}\gamma^{G}\gamma^{A}\gamma$.[7,17,18] We have analyzed 20 $\gamma\gamma$, 23 $-\gamma$ and 9 $\gamma\gamma\gamma$ chromosomes with Pst I, and in every case these were $^{G}\gamma^{A}\gamma$, $-^{A}\gamma$, and $^{G}\gamma^{G}\gamma^{A}\gamma$, respectively. The $^{G}\gamma^{G}\gamma$ chromosome reported in American blacks and Chinese[17,19] was not found in this population. These single and triple gene types are to be expected since unequal cross-overs in the much larger segment of the γ homology block that lies 5′ to codon 136[15] will always produce single-$^{A}\gamma$ and triplicated $^{G}\gamma^{G}\gamma^{A}\gamma$ arrangements. Likewise, a further such cross-over between $^{G}\gamma^{G}\gamma^{A}\gamma$ and either a $^{G}\gamma^{G}\gamma^{A}\gamma$ or $^{G}\gamma^{A}\gamma$ chromosome could produce a quadruple $^{G}\gamma^{G}\gamma^{G}\gamma^{A}\gamma$ chromo-

Table 1. Frequency of the Various γ Genotypes in 852 Melanesians From Different Islands in Vanuatu and From New Caledonia

Islands	No.	$\gamma\gamma/\gamma\gamma$	$-\gamma/\gamma\gamma$	$\gamma\gamma\gamma/\gamma\gamma$	$-\gamma/\gamma\gamma\gamma$	$\gamma\gamma\gamma/\gamma\gamma\gamma$	$-\gamma/-\gamma$	$\gamma\gamma\gamma\gamma/\gamma\gamma$	$-\gamma$ (%)	$\gamma\gamma\gamma$ (%)
Espiritu Santo	82	60	16	5	0	0	1	0	11.0	3.0
Maewo	168	114	24	21	7	1	1	0	9.8	8.9
Pentecost	136	120	4	11	0	0	1	0	2.2	4.0
Emae	68	49	16	3	0	0	0	0	11.8	2.2
Central	209	185	11	11	0	1	0	1	2.6	3.1
Tanna	48	45	2	1	0	0	0	0	2.1	1.1
Futuna	54	41	13	0	0	0	0	0	12.5	0.0
Aneityum	57	46	9	1	0	0	1	0	9.8	0.9
New Caledonia	30	25	1	3	0	1	0	0	1.7	8.3
Total	852	685	96	56	7	3	4	1	6.5	3.9

Islands are listed from north to south. The Central group includes the Shepherd group of islands (excluding Emae) as well as Efate, where all the umbilical cord bloods were collected.

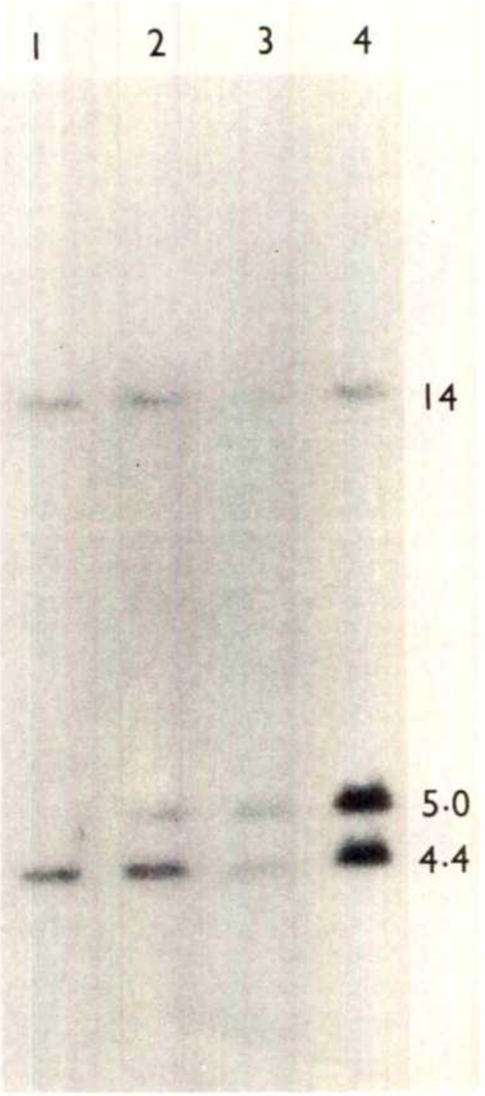

Fig 3. Restriction fragments seen in individuals of the following genotypes following Sph I digestion and hybridization with the γ probe: lane 1, $\gamma\gamma/\gamma\gamma$; lane 2, $\gamma\gamma\gamma/\gamma\gamma$; lane 3, $\gamma\gamma\gamma\gamma/\gamma\gamma$; lane 4, $\gamma\gamma\gamma/\gamma\gamma\gamma$. In lane 2 the 5-kb band is half the intensity of the 4.4-kb band, whereas in lanes 3 and 4 it is equal in intensity to the 4.4-kb band because two additional γ genes are present.

some. Analysis of the individual with the $\gamma\gamma\gamma\gamma$ chromosome using Pst I confirmed that the arrangement in her case is, in fact, ${}^{G}\gamma{}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$ (data not shown).

${}^{G}\gamma$:${}^{A}\gamma$ ratios in cord bloods. The usual ratio of ${}^{G}\gamma$- to ${}^{A}\gamma$-chains in HbF is approximately 7:3 at birth.[20] We measured this ratio in cord bloods from three heterozygotes for a single-γ gene, six heterozygotes for a triple-γ gene, one homozygote $\gamma\gamma\gamma/\gamma\gamma\gamma$, and the quadruple-$\gamma$ gene carrier. The results (Fig 4) indicated that the extra ${}^{G}\gamma$ gene on ${}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$ chromosomes raised the ${}^{G}\gamma$:${}^{A}\gamma$ ratio as would be expected if all three genes on the chromosome were expressed.[17] Similarly, the very high ratios in the ${}^{G}\gamma{}^{G}\gamma{}^{A}\gamma/{}^{G}\gamma{}^{G}\gamma{}^{A}\gamma$ and ${}^{G}\gamma{}^{G}\gamma{}^{G}\gamma{}^{A}\gamma/{}^{G}\gamma{}^{A}\gamma$ individuals are consistent with the expression of all ${}^{G}\gamma$ genes in these cases. The ratios of about 0.52 in the single-${}^{A}\gamma$ gene heterozygote are lower than normal as would be expected following the loss of one ${}^{G}\gamma$ gene. Judging by this ratio, the residual ${}^{A}\gamma$ gene on the single-gene chromosome is still expressed at approximately the level of a normal ${}^{A}\gamma$ gene on a two-gene chromosome. If it were expressed at the level of a ${}^{G}\gamma$ gene at this gestational stage, the overall ${}^{G}\gamma$:${}^{A}\gamma$ ratio should be less than 0.5 as has been reported in Asian cases.[21] It seemed possible that this apparent difference in the level of expression of the single-${}^{A}\gamma$ gene between Asian and Melanesian infants might be due to the presence in the Asian cases and absence in Melanesians of a putative regulatory sequence 5′ to the gene that may be detected by the restriction enzyme Xmn I. The presence of this Xmn I site at position −158, 5′ to the ${}^{G}\gamma$ gene, correlates with increased ${}^{G}\gamma$:${}^{A}\gamma$ ratios, at least in individuals with some erythropoietic stress, and it has been proposed that this sequence might itself increase expression of the adjacent gene.[22] However, since all three Melanesians of genotype $-{}^{A}\gamma/{}^{G}\gamma{}^{A}\gamma$ with ${}^{G}\gamma$:${}^{A}\gamma$ ratios greater than 0.5 had the polymorphic Xmn I site 5′ to the $-{}^{A}\gamma$ gene (data not shown), it would appear that the apparent population difference in this ratio is not explained by an effect of this Xmn I site.

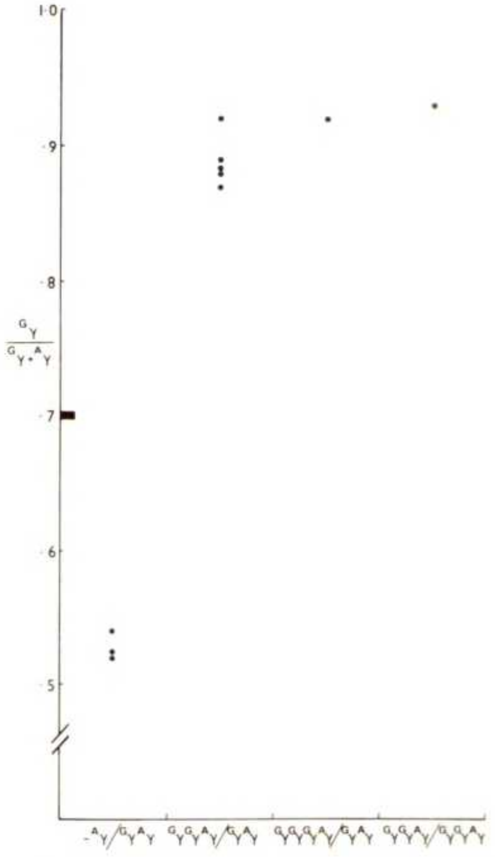

Fig 4. Proportions of ${}^{G}\gamma$ in HbF from cord bloods of different genotypes. The proportion seen in individuals of normal (${}^{G}\gamma{}^{A}\gamma/{}^{G}\gamma{}^{A}\gamma$) genotype, ~.7, is shown as a black bar.

Since the extra γ gene on triplicated γ chromosomes is expressed, we measured Bart's Hb (γ_4) levels in cord bloods from individuals heterozygous and homozygous for the $\gamma\gamma\gamma$ arrangement. One $\gamma\gamma\gamma$ heterozygote had a normal ($\alpha\alpha/\alpha\alpha$) α genotype and no detectable Bart's Hb as measured by

Table 2. HbF Levels With Different γ Genotypes

Genotype	No.	Percentage
$\gamma\gamma/\gamma\gamma$	98	.28 ± .12
$-\gamma/\gamma\gamma$	19	.21 ± .06
$\gamma\gamma\gamma/\gamma\gamma$	17	.36 ± .21
$-\gamma/\gamma\gamma\gamma$	6	.31 ± .09
$-\gamma/-\gamma$	2	.65, .17
$\gamma\gamma\gamma/\gamma\gamma\gamma$	2	.51, .25

HbF values associated with the various γ genotypes seen in adults from Island Melanesia. For the four larger genotype groups the results are presented as mean ± SD. The mean HbF level for the $\gamma\gamma/\gamma\gamma$ genotype was significantly different from both the $-\gamma/\gamma\gamma$ genotype ($P < .005$) and the $\gamma\gamma\gamma/\gamma\gamma$ genotype ($P < .05$), using Student's t test on log-transformed data.

cellulose acetate electrophoresis. Three $\gamma\gamma\gamma$ heterozygotes were also heterozygous for α^+-thalassemia ($-\alpha^{3.7}/\alpha\alpha$) and had, on the average, 1.7% Bart's Hb, a typical level for this α genotype. The $\gamma\gamma\gamma/\gamma\gamma\gamma$ homozygote had 1.8% Bart's Hb and the $-\alpha/\alpha\alpha$ genotype. The individual heterozygous for a quadruple-γ gene ($\gamma\gamma\gamma\gamma/\gamma\gamma$) had a single-$\alpha$ gene deletion ($-\alpha^{3.7}/\alpha\alpha$) and 1.26% Bart's Hb. Similarly, analysis of seven heterozygotes for single-γ gene deletions showed no alteration in the expected Bart's Hb level. We conclude that changes in copy number of the fetal γ-globin genes do not significantly affect the Bart's Hb level in cord bloods. Hence these γ gene variants should not decrease the effectiveness of cord blood Bart's Hb levels as a method of detecting α-thalassemia.

HbF levels in adults with two to six γ genes. We have previously reported heterozygotes for the triple-γ gene arrangement.[7] The HbF levels in one family were raised, but it seemed that this might be due to coinheritance of a gene for modifying HbF production. In a large Indian family, heterozygotes for the triple-γ gene chromosome had normal HbF levels.[18] There has been no report of HbF levels in adults homozygous for single-γ gene deletions or triple-γ genes. In this survey we have measured the HbF levels of 17 individuals of genotype $\gamma\gamma\gamma/\gamma\gamma$, 19 of genotype $-\gamma/\gamma\gamma$, as well as two homozygotes for triple-γ genes, two homozygotes for single-γ genes, and six double heterozygotes (Table 2). Heterozygotes for the triple-γ gene had slightly higher and heterozygotes for the single-γ gene slightly lower mean HbF levels than normal individuals. Homozygotes for both triple- and single-γ genes and double heterozygotes all had HbF levels in the normal range as well as normal blood counts. So, although extra γ genes appear to be expressed at birth, they do not prevent the fetal to adult Hb switch, and HbF levels in adult life are in the normal range.

We have also followed up the child in the cord blood survey found to have a quadruple-γ gene chromosome. A family study performed when she was 2 years old is summarized in Table 3. Her father also has a quadruple-γ gene and is heterozygous for the α^+-thalassemia $\text{HbJ}^{\text{Tongariki}}$ determinant ($-\alpha^J/\alpha\alpha$).[23] His HbF and HbA_2 levels were in the normal range, but within the family the two individuals with the $\gamma\gamma\gamma\gamma$ gene have higher HbF levels than the others. This may mean that the extra γ genes are being expressed but not at a level that would raise the HbF percentage to above the normal range. The red cell indexes in the father and two daughters are low even for carriers of α^+-thalassemia.[6] It is possible that this may be due to a nondeletion α^+-thalassemia determinant segregating in this family.

Restriction enzyme haplotypes in Vanuatu. To investigate possible mechanisms of recombination that give rise to loss and amplification of γ genes we have haplotyped polymorphic restriction enzyme sites[24] in the β complex of both normal and variant chromosomes in this population. Table 4 shows the normal haplotypes of 69 Melanesians from Vanuatu. These are divided into 5′ and 3′ linkage groups because the DNA segment between these two regions has been shown to be a recombination hotspot.[25-27] In the 3′ block we have identified a new polymorphic Hind III restriction enzyme site 11 kb 3′ to the β-globin gene that produces an 8-kb band with the pRK 29 probe (Fig 1). This polymorphism has been found in Melanesian and Polynesian individuals and was always associated with a 7.0-kb Hpa I β fragment. The normal 5′ haplotypes are generally similar in type and frequency to those in most Caucasian and Oriental populations[24,28] except that the relatively common haplotype $-++-+$ is not present. Clearly, there are two predominant 5′ haplotypes, $+----$ and $-+-++$, which will be denoted $+[--]--$ and $-[+-]++$ to emphasize the second and third sites that are in the γ genes.

Haplotypes of single, triple, and quadruple γ genes. We have determined the 5′ haplotypes (which will be simply termed 'haplotypes') of chromosomes with triple- and single-γ genes. There are two haplotypes associated with each arrangement as shown in Table 5. Interestingly, these are the haplotypes that would be expected following independent intrachromosomal recombination events on chromosomes with the two common haplotypes previously described.

Table 3. Hematologic Data on the $\gamma\gamma\gamma\gamma$ Genotype Family

Pedigree No.	Age	Sex	Hb	MCV	MCHC	MCH	Ferritin	HbF	HbA_2	α Genotype	γ Genotype
I.1	adult	F	13.3	84.2	32.7	27.6	N	0.33%	2.5%	$\alpha\alpha/\alpha\alpha$	$\gamma\gamma/\gamma\gamma$
I.2	adult	M	12.5	67.3	30.7	20.7	N	0.49%	3.2%	$-\alpha^J/\alpha\alpha$	$\gamma\gamma\gamma\gamma/\gamma\gamma$
II.1	11 yr	F	13.2	80.6	31.8	25.7	N	0.33%	1.3%	$\alpha\alpha/\alpha\alpha$	$\gamma\gamma/\gamma\gamma$
II.2	6 yr	F	12.6	67.3	32.2	21.7	N	0.39%	2.6%	$\alpha\alpha/\alpha\alpha$	$\gamma\gamma/\gamma\gamma$
II.3	2 yr	F	11.1	66.2	30.7	20.4	N	0.55%	1.1%	$-\alpha^J/\alpha\alpha$	$\gamma\gamma\gamma\gamma/\gamma\gamma$

Abbreviations: MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCH, mean corpuscular hemoglobin; N, normal.

Results of hematologic and DNA analyses on the family with a $\gamma\gamma\gamma\gamma$ chromosome. The propositus (II.3) and her father both have lower MCH values than typical α^+-thalassemia heterozygotes6 despite normal levels of ferritin. II.1, a half sib of II.2 and II.3 has a different father who was not studied.

Table 4. Frequencies of Different 5′ and 3′ Haplotypes on β^{A} Chromosomes in Vanuatu

5′ Haplotype	No.	3′ Haplotype	No.
+ − − − −	51	+ + −	14
− + − + +	13	− − +	11
− + + + +	3	+ + +	9
+ − − + +	2	+ − +	7
		+ V +	6

The five sites used for the 5′ haplotype are (1) Hinc II 5′ to ϵ, (2) Hind III in $^{G}\gamma$, (3) Hind III in $^{A}\gamma$, (4) and (5) the polymorphic Hinc II sites within and 3′ to the $\psi\beta$ gene, respectively. The three sites used for the 3′ haplotype are (1) Ava II in the β gene, (2) Hind III 7 kb 3′ to the β, and (3) Bam HI 3′ to β. V indicates a new polymorphic Hind III site approximately 11 kb 3′ to the β gene. Other possible haplotypes combinations that are not listed were not found.

Recombination on the +[− −]− − haplotype should give rise to triple-γ +[− − −]− − chromosomes and single +[−]− − chromosomes. Similarly, recombination on the −[+ −]+ + chromosome would produce −[+ + −]+ + triple and −[−]+ + single chromosomes (Fig 5), as observed. These resultant haplotypes are created only if the unequal cross-overs occur 5′ to the polymorphic Hind III site in intron 2. Although sequence analysis will be required for final confirmation, this seems likely for several reasons. Crossing-over takes place 5′ to the Pst I site in exon III (see previous material) because all $\gamma\gamma\gamma$ chromosomes are $^{G}\gamma^{G}\gamma^{A}\gamma$ and all -γ chromosomes, $^{A}\gamma$. By far the largest region of complete homology between $^{G}\gamma$ and $^{A}\gamma$ lies 5′ to the Hind III sites, and previously described cross-overs producing triple- and single-γ genes are all compatible with this model. Furthermore, using the restriction enzyme Xmn I, which detects a polymorphism at the 5′ boundary of this homology block (see previous material, Fig 1), we found that all cross-overs have taken place 3′ to this site (data not shown). Similarly, the quadruple-γ chromosome of haplotype +[− − − −]− − may have been produced by an intrachromosomal recombination event on a triple–γ gene chromosome of haplotype +[− − −]− − (Fig 6).

False haplotypes resulting from single– and triple–γ gene chromosomes. Since haplotype data are being increasingly used both for prenatal diagnosis of β-globin gene disorders[29] and population studies,[28] it should be noted that single– and triple–γ gene chromosomes can produce band patterns with Hind III that may be interpreted incorrectly and produce "false" haplotypes. For example, an individual with the genotype $\gamma\gamma$/-γ and haplotypes +[− −]− −/−[−]+ + will produce γ bands following Hind III digestion of 8.0 and 3.2 kb. Hence, unless loss of a single-γ gene has been detected separately, this individual would be typed as +[− −]− −/−[− −]+ +. The haplotype −[− −]+ + is very rare in all populations tested to date and in this case is a false haplotype. Similarly, an individual of genotype $\gamma\gamma\gamma/\gamma\gamma$ and haplotype +[− −]− −/−[+ + −]+ + produces Hind III γ bands of 8.0, 7.2, 3.2 and 2.7 kb that would normally (and here incorrectly) be interpreted as +[− −]− −/−[+ +]+ +. We conclude that for determination of 5′ haplotypes with certainty, the number of γ genes on the chromosome should be determined separately using, for example, a Bgl II digest.

Table 5. 5′ Haplotypes of Single-, Triple-, and Quadruple-γ Gene Chromosomes

γ Genotype	No. of Chromosomes	Haplotypes
−γ	3	+[−]− −
−γ	9	−[−]+ +
$\gamma\gamma\gamma$	6	+[− − −]− −
$\gamma\gamma\gamma$	6	−[+ + −]+ +
$\gamma\gamma\gamma\gamma$	1	+[− − − −]− −

The sites are as indicated in Table 3 with the Hind III sites in the γ genes within square brackets and the Hinc II sites near the ϵ and $\psi\beta$ genes to the left and right of these brackets, respectively. The number of chromosomes of each type analyzed is also shown.

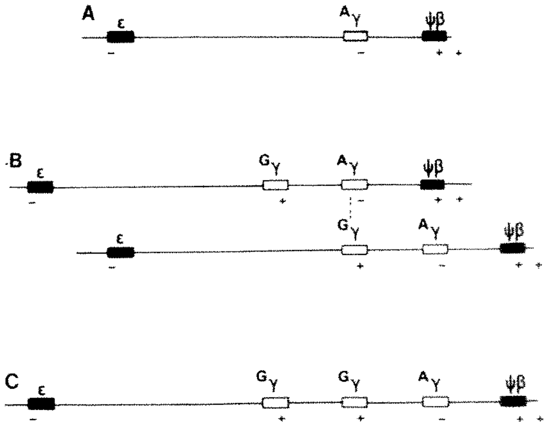

Fig 5. Unequal meiotic crossing-over producing (A) single–γ and (C) triple–γ gene chromosomes. (B) Crossing-over takes place in the 5′ half of the misaligned genes to produce the variant chromosomes that show different restriction enzymes haplotypes from the original $\gamma\gamma$ chromosome (see text). The five restriction enzyme site polymorphisms shown are those described in the legend to Table 4.

DISCUSSION

DNA analysis of γ-globin genotypes has revealed a high prevalence of single- and triple-γ genes in Vanuatu. Evidence from smaller DNA surveys[14] and analysis of $^{G}\gamma$:$^{A}\gamma$ ratios[21,30] suggest that these rearrangements are significantly less frequent in Europeans, American blacks, Asian Indians, and Chinese, although the $\gamma\gamma\gamma$ chromosome is common in some Japanese.[9] Although triplications of normally duplicated

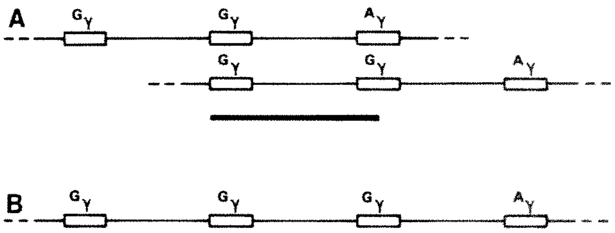

Fig 6. Proposed model of unequal crossing-over between (A) two misaligned sister chromatids of $\gamma\gamma\gamma$ genotype that produces (B) the $\gamma\gamma\gamma\gamma$ chromosome as well as a $\gamma\gamma$ chromosome. The large region of complete sequence homology between the misaligned sister chromatids is indicated by the black bar.

human globin genes have been reported from several populations, the finding of a quadruple-γ gene chromosome in Vanuatu and Japan[9] is, to our knowledge, the only example of a quadruplication of normally duplicated human genes.

These γ gene rearrangements add to the remarkable variety of novel globin genes found in Melanesian populations. For example, the α-chain variant HbJ$^{\text{Tongariki}}$ is associated with an α^+-thalassemia deletion that is confined to the Southwest Pacific,[31] and in north coastal Papua New Guinea a different α gene deletion ($-\alpha^{4.2}$) has gone almost to fixation.[32] Several ζ-globin gene variants[33] and localized clusterings of β-thalassemia and HbG$^{\text{Philadelphia}}$ have also been discovered in these islands.[6,23] The interisland variation in the frequency of these as well as the γ gene variants reported here emphasize that historically these islanders must have remained relatively isolated in spite of their proximity. In view of the hematologic similarities of individuals of different γ genotype it would appear that these γ gene variants are probably selectively neutral and that the interisland differences and the overall high prevalence reflect genetic drift. With a population of only 115,000 and more than 70 islands Vanuatu provides a likely setting for large gene frequency differences resulting from drift; there are other examples of the importance of this phenomenon in the Pacific.[34] However, the relative contributions of various factors such as small founder population size, variation in mating structure, and lack of migration to the overall amount of genetic drift remain to be defined. We have previously shown that α-globin gene variants in Melanesia can be useful as anthropologic markers in analyzing population affinities and reconstructing prehistoric migration routes.[31] The high frequency of variant γ genotypes in Melanesia and, in particular, the high frequency of single-γ genes in the ethnically Polynesian populations suggest that γ genotype surveys on other Pacific islands should be of similar interest.

Because of the high frequency of the $-\gamma$ and $\gamma\gamma\gamma$ chromosomes in this population we have been able to study the phenotype of both these variants in the heterozygous and homozygous states. In particular, we have measured HbF levels in adults and $^{G}\gamma$:$^{A}\gamma$ ratios in cord bloods to study the expression of the single- and triple-γ gene arrangements and their effect, if any, on the fetal to adult hemoglobin switch. The previously described adult heterozygote with a single-γ gene, $-\gamma/\gamma\gamma$, had a normal HbF level, and this is true of both heterozygotes and homozygotes. The slightly lower mean levels of HbF in adult heterozygotes may reflect reduced γ-chain output from the $-\gamma$ chromosome. Our data on two homozygotes and 17 heterozygotes for the $\gamma\gamma\gamma$ chromosome show that although on average these individuals have slightly higher HbF levels than normal individuals, the difference is small and does not produce abnormally high HbF levels. Such a limited elevation in γ-chain output is consistent with the elevated $^{G}\gamma$:$^{A}\gamma$ ratios seen in newborns with an extra $^{G}\gamma$ gene. Hence each gene appears to be expressed on $\gamma\gamma\gamma$ chromosomes as has been shown to be the case for triplicated α-globin genes.[35] The individual with the quadruple-γ gene and her father, who also has this arrangement, have normal HbF and HbA$_2$ levels. The high $^{G}\gamma$:$^{A}\gamma$ ratio of 0.93 in cord blood suggests that all three $^{G}\gamma$ genes on the quadruple chromosome are expressed. However, all four γ genes are switched off by the age of 2 years, and they do not lead to a raised adult HbF level. Both child and father have unusually low MCH and MCV values even for α^+-thalassemia heterozygotes. This may be due to additional nondeletion α^+-thalassemia determinants in the family, although these appear to be relatively uncommon in Vanuatu. However, a more intriguing possibility, which we cannot as yet exclude, is that expression of the β-globin gene on this chromosome is in some way reduced by the presence of two additional γ genes. The observation that expression of the $^{A}\gamma$ gene on the Japanese chromosome with four γ genes is reduced relative to the $^{A}\gamma$ gene on the homologous ($\gamma\gamma$) chromosome[9] is consistent with this mechanism.

The population data on the haplotypes of the various γ genotypes allow some inferences to be made on the probable type of recombination event that produces γ gene rearrangements. If we know the frequencies of the $+[--]--$ and $-[+-]++$ haplotypes in the population in which the recombination events leading to single- and triple-γ genes occurred, we may calculate the probability that the observed haplotypes were produced, assuming that only interchromosomal recombination is possible. At least four recombination events are required to account for the observed $\gamma\gamma\gamma$ and $-\gamma$ chromosomes (two different haplotypes for each) since the probability of both products of a single meiotic event (ie, the resulting $\gamma\gamma\gamma$ and $-\gamma$ chromosomes) each being found in the next generation is negligible in man. Using approximate frequencies of .75 and .25 for each of the two predominant haplotypes (Table 4) the probability that interchromosomal cross-overs at meiosis would have occurred on four occasions in an individual with an identical haplotype on the allelic chromosome is small (pxpxqxq $= .75 \times .75 \times .25 \times .25 = .035$). Given the low prevalence ($<1\%$) of triplicated γ genes in most populations, it is surprising that we should have found one quadruple-γ gene in a survey of less than 2,000 chromosomes. This may be explained in part by the local high frequences ($>8\%$) of $\gamma\gamma\gamma$ arrangements on some islands in Vanuatu. However, it is also possible that triplicated genes are more likely to misalign than duplicate genes because of the larger region of homology between misaligned triplicated genes (Fig 6). The haplotype of the $\gamma\gamma\gamma\gamma$ chromosome, $+[----]--$, is completely consistent with its formation from an unequal cross-over between misaligned sister chromatids of the $+[---]---$ haplotype, although an interchromosomal recombination event cannot be excluded.

Hence, interchromosomal recombination is unlikely to be the sole mechanism leading to changes in γ gene copy number. Our recent observation of a triplicated ζ-globin gene produced by interchromosomal recombination[33] does not necessarily conflict with these results because, by using flanking polymorphisms to classify globin gene rearrangements, only interchromosomal events can be positively identified. Therefore, it may be that, as in yeast,[36] genetic recombination events in man are predominantly intrachromosomal. The observed stability of 5′ β-globin haplotypes for long periods of human evolution[28] also suggests that interchromosomal recombination, at least within the 5′ segment of the β-globin gene cluster, must be relatively infrequent. Intrachromosomal recombination to produce a single-γ gene could happen in at least two ways. There could either be an unequal cross-over between misaligned sister chromatids, an example of sister chromatid exchange, or an intrachromatid deletion could occur. We cannot distinguish between these two possibilities, but, clearly, triplicated γ genes could only be generated intrachromosomally by the former, sister chromatid exchange, model.

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REFERENCES

1. Weatherall DJ, Clegg JB: Thalassemia revisited. Cell 29:7, 1982
2. Collins FS, Weissman SM: The molecular genetics of human hemoglobin. Prog Nucleic Acids Res Mol Biol 31:315, 1984
3. Jones RW, Old JM, Trent RJ, Clegg JB, Weatherall DJ: Major rearrangement in the human β globin gene cluster. Nature 291:39, 1980
4. Vanin EF, Henthorn PS, Kioussis D, Grosveld F, Smithies O: Unexpected relationships between four large deletions in the human β-globin gene cluster. Cell 35:701, 1983
5. Higgs DR, Weatherall DJ: Alpha-Thalassaemia, in Piomelli S, Yachnin S (eds): Current Topics in Hematology, vol 4. New York, Liss, 1983, p 37
6. Bowden DK, Hill AVS, Higgs DR, Weatherall DJ, Clegg JB: The relative roles of genetic factors, dietary deficiency and infections in anaemia in the southwest Pacific. Lancet, 2:1025, 1985
7. Trent RJ, Bowden DK, Old JM, Wainscoat JS, Clegg JB, Weatherall DJ: A novel rearrangement of the human β-like globin gene cluster. Nucleic Acids Res 9:6723, 1981
8. Sukumaran PK, Nakatsuji T, Gardiner MB, Reese AL, Gilman JG, Huisman THJ: Gamma thalassemia resulting from the deletion of a γ-globin gene. Nucleic Acids Res 11:4635, 1983
9. Harano K, Harano T, Kutlar F, Huisman THJ: γ-globin triplication and quadruplication in Japanese newborns. FEBS Lett 190:45, 1985
10. Weatherall DJ (ed): The Thalassaemias. Methods in Haematology. London, Churchill Livingstone, 1983
11. Wood WG, Weatherall DJ, Clegg JB, Hamblin TJ, Edwards JH, Barlow AM: Heterocellular hereditary persistence of fetal haemoglobin (heterocellular HPFH) and its interaction with β thalassemia. Br J Haematol 36:461, 1977
12. Tuan D, Feingold E, Newman M, Weissman SM, Forget BG: Different 3′ end points of deletions causing $\delta\beta$-thalassemia and hereditary persistence of fetal haemoglobin: Implications for the control of γ-globin gene expression in man. Proc Natl Acad Sci USA 80:6937, 1983
13. Bellwood P: Man's conquest of the Pacific. London, Collins, 1978
14. Trent RJ: Genetic mechanisms of some forms of thalassaemia. Doctoral thesis, University of Oxford, England, 1983
15. Shen S, Slightom JL, Smithies O: A history of the human fetal globin gene duplication. Cell 26:191, 1981
16. Slightom JL, Blechl AE, Smithies O: Human fetal ${}^{G}\gamma$- and ${}^{A}\gamma$-globin genes: Complete nucleotide sequences suggest that DNA can be exchanged between these duplicated genes. Cell 21:627, 1980
17. Harano T, Huisman THJ, Zeng YT, Huang SZ: Two arrangements of the human fetal globin genes may be responsible for high ${}^{G}\gamma$ values in Chinese newborns: $-{}^{G}\gamma-{}^{G}\gamma-\delta-\beta$ and $-{}^{G}\gamma-{}^{A}\gamma{}^{G}\gamma-{}^{A}\gamma-\delta-\beta$. Am J Hematol 19:349, 1985
18. Thein SL, Hill FGH, Weatherall DJ: Haematological phenotype of the triplicated γ-globin gene arrangement. Br J Haematol 57:349, 1984
19. Powers PA, Altay C, Huisman THJ, Smithies O: Two novel arrangements of the human fetal globin genes: ${}^{G}\gamma-{}^{G}\gamma$ and ${}^{A}\gamma-{}^{A}\gamma$. Nucleic Acids Res 12:7023, 1984
20. Schroeder WA, Huisman THJ, Shelton JR, Shelton JB, Kleihauer EF, Dozy AM, Robberson B: Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. Proc Natl Acad Sci USA 60:537, 1968
21. Zeng Y, Huang S-Z, Nakatsuji T, Huisman THJ: $-{}^{G}\gamma{}^{A}\gamma$-thalassemia and γ-chain variants in Chinese newborn babies. Am J Hematol 18:235, 1985
22. Gilman JB, Huisman THJ: DNA sequence variation associated with elevated fetal ${}^{G}\gamma$ globin production. Blood 66:783, 1985
23. Bowden DK, Pressley L, Higgs DR, Clegg JB, Weatherall DJ: α-Globin gene deletions associated with Hb $J^{Tongariki}$. Br J Haematol 51:243, 1982
24. Orkin SH, Kazazian HH: The mutation and polymorphism of the human β-globin gene and its surrounding DNA. Annu Rev Genet 18:131, 1984
25. Antonarakis SE, Boehm CD, Giardina PJV, Kazazian HH: Non-random association of polymorphic restriction sites in the β globin gene cluster. Proc Natl Acad Sci USA 79:137, 1982
26. Gerhard DS, Kidd KK, Kidd JR, Egeland JA, Housman DE: Identification of a recent recombination event within the human β-globin gene cluster. Proc Natl Acad Sci USA 81:7875, 1984
27. Old JM, Heath C, Fitches A, Thein SL, Jeffreys AJ, Petrou M, Modell B, Weatherall DJ: Meiotic recombination between two polymorphic restriction sites within the β-globin gene cluster. J Med Genet 23:14, 1986
28. Wainscoat JS, Hill AVS, Boyce A, Flint J, Hernandez M, Thein SL, Old JM, Lynch J, Falusi Y, Weatherall DJ, Clegg JB: Evolutionary relationships of human populations from an analysis of nuclear DNA polymorphisms. Nature 319:491, 1986
29. Old JM, Petrou M, Modell B, Weatherall DJ: Feasibility of antenatal diagnosis of β thalassemia by DNA polymorphisms in Asian Indian and Cypriot populations. Br J Haematol 57:255, 1984
30. Huisman THJ, Reese AL, Gardiner MB, Wilson JB, Lam H, Reynolds A, Nagle S, Trowell P, Zeng Yi-tao, Huang Shu-zheng, Sukumaran PK, Miwa S, Efremov GD, Petkov G, Sciarratta GV, Sansone G: The occurrence of different levels of ${}^{G}\gamma$ chain and of the ${}^{A}\gamma^{T}$ variant of fetal hemoglobin in newborn babies from several countries. Am J Hematol 14:133, 1983
31. Hill AVS, Bowden DK, Trent RJ, Higgs DR, Oppenheimer SJ, Thein SL, Mickleson KNP, Weatherall DJ, Clegg JB: Melanesians and Polynesians share an unique α-thalassemia mutation. Am J Hum Genet 37:571, 1985
32. Oppenheimer SJ, Higgs DR, Weatherall DJ, Barker J, Spark RA: α thalassemia in Papua New Guinea. Lancet 1:424, 1984
33. Hill AVS, Nicholls RD, Thein SL, Higgs DR: Recombination within the human embryonic ζ globin locus. A common ζ-ζ

chromosome produced by gene conversion of the $\psi\zeta$ gene. Cell 42:809, 1985

34. Kirk RL: Language, genes and people in the Pacific, in Eriksson AN, Forsius H, Nevanlinna HR, Workman PL, Norio PK (eds): Population Structure and Genetic Disorders. Orlando, Fla, Academic, p 113, 1980

35. Higgs DR, Clegg JB, Weatherall DJ, Serjeant BE, Serjeant GR: Interaction of the $\alpha\alpha\alpha$ globin gene haplotype and sickle haemoglobin. Br J Haematol 58:671, 1984

36. Mikus MD, Petes TD: Recombination between genes located on non-homologous chromosomes in *Saccharomyces cerevisiae.* Genetics 101:369, 1982

T Cell–Derived Migration-Inhibitory Factor and Colony-Stimulating Factor Share Common Structural Elements

By Tsutomu Kawaguchi, David W. Golde, Alma Mednis, Noelle Bersch, Steve Clark, and Heinz G. Remold

Migration-inhibitory factor (MIF) is a lymphokine that acts to localize mononuclear phagocytes (monocytes and macrophages) and perhaps to activate them. Mo cells are a human T cell leukemia virus II–infected T cell line previously shown to secrete large quantities of MIF upon stimulation with phytohemagglutinin and phorbol myristate acetate. MIF was purified from Mo cell–conditioned medium by gel filtration, phenyl-Sepharose affinity chromatography, isoelectrofocusing, and reverse-phase high-performance liquid chromatography (RP-HPLC). Overall purification was 6,000-fold. The purified MIF fraction was found to display potent colony-stimulating factor (CSF) activity when assayed on human bone marrow cells. The double peak of MIF activity as shown by C 18–RP-HPLC coincided with the double peak of CSF activity. A monoclonal antibody selected for its anti-MIF activity absorbed both the CSF and the MIF activity. These findings indicate that MIF and CSF are either identical molecules or closely related molecules with common structural elements.
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LYMPHOKINES are glycoprotein hormones produced by antigen-sensitized and -stimulated lymphocytes that play an essential role in regulating cellular immune responses. Lymphokines function prominently in the modulation of effector cell number and function. Several lymphokines are directed at the macrophage including migration-inhibitory factor (MIF), macrophage-activating factor(s), and colony-stimulating factors (CSF). MIF produced by human blood lymphocytes has been partially purified and characterized.[1] We previously described the production of MIF by mitogenic stimulation of the human T cell leukemia virus II (HTLV)-II–infected T lymphocyte cell line Mo.[2] Stimulated Mo cells produce MIF at levels tenfold higher than stimulated peripheral blood lymphocytes.[2] Here, we describe that highly purified MIF from Mo cell–conditioned medium after 6,000-fold purification also has CSF activity inducing the proliferation of bone marrow myeloid precursors.

MATERIALS AND METHODS

Production of Mo-MIF and recombinant GM-CSF. Mo cells[3,4] were grown in Iscove's medium[5] with 20% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. For the production of Mo-MIF, 1×10^6 cells/mL were cultured in serum-free medium with 300 μg/mL phytohemagglutinin M (PHA-M, Difco, Detroit, Mich) and 1.5×10^{-7} mol/L phorbol myristic acetate for 96 hours at 37 °C. After removal of the cells by centrifugation, the media (25 μg protein/mL) were concentrated 200-fold by vacuum dialysis. Recombinant GM-CSF was obtained from conditioned medium from Cos I cells transfected with pCSF-1, a GM-CSF–encoding cDNA-clone.[6]

Purification of Mo-MIF. Concentrated conditioned media from 0.5- to 1.0-L cultures were fractionated on Sephadex G-100 columns (2.5×100 cm) in phosphate-buffered saline (PBS). The pooled MIF-containing fractions containing molecules with an apparent molecular weight (mol wt) of 30,000 to 60,000 which were 14-fold purified relative to conditioned medium were concentrated to 25 to 50 mL, adjusted to 40% ammonium sulfate in 20 mmol/L Tris-HCl buffer (pH 8.0) containing 0.3 mmol/L EDTA, 20 mmol/L NaCl and 5.3 mmol/L $MgCL_2$ (TBS), and applied to a 1.5×5.5-cm phenyl-Sepharose column. After washing with 50 mL of 40% ammonium sulfate–TBS, the column was eluted with a 100-mL linear gradient of decreasing ammonium sulfate (40% to 0%) and increasing ethylene glycol (0% to 50%) in TBS. MIF eluted at 20% ammonium sulfate and 25% ethylene glycol. The MIF-containing fractions were adjusted to 40% ammonium sulfate and rechromatographed on phenyl-Sepharose, resulting in a 190-fold total purification and an apparent yield of 90%. The MIF-containing fractions were subjected to isoelectrofocusing in a sucrose-stabilized ampholyte gradient from pH 2.5 to 6.5.[7] To monitor protein in subsequent steps, the partially purified MIF was radiolabeled by reductive methylation with ^{14}C-formaldehyde to a specific radioactivity of 1.4×10^4 dpm/μg protein as determined by the method of Lowry et al.[8] Reductive methylation[9] was carried out in a 1-mL reaction volume containing 0.1 to 0.2 mg protein, 2 mmol/L ^{14}C-formaldehyde (53 mCi/mmol, New England Nuclear Corp, Boston), 20 mmol/L $NaCNBH_3$ (Aldrich Biochemical Corp, Milwaukee), and 0.1 mol/L HEPES buffer. After one hour at room temperature, the mixture was filtered over a Sephadex G-25 column (1×5 cm) equilibrated with PBS. Rechromatography of the radioactively labeled material in the presence of 0.01% bovine serum albumin yielded a single peak of MIF activity[2] with an isoelectric point (pI) of 4.8 to 5.5, a yield of 28%, and a 2,600-fold total purification (50 μg/mL protein).

The pooled ^{14}C-labeled MIF-containing fractions from the second preparative isoelectrofocusing (IEF) column were dialyzed against 0.04% trifluoroacetic acid (TFA) for one hour and applied to a C-18 column (Waters Assoc, Milford, Mass) using a Spectrophysics high-performance liquid chromatography (HPLC) system with 0.04% TFA in water as the primary solvent. After the collection of 15 mL, a linear 0% to 95% ethanol gradient in 0.04% TFA was applied. One-milliliter fractions were collected into tubes containing 10 μg bovine serum albumin and dialyzed against TBS. The amount of protein in the active fractions (5 μg/mL) was assessed by determining the radioactivity.

Production of a monoclonal antibody (MoAb) against Mo-MIF. For use as an immunogen, MIF from 1 L of Mo-conditioned medium (25 mg protein, 1,400 units MIF, see the following) was partially purified in a 57% yield by Sephadex G-100 gel filtration

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and isoelectrofocusing steps[7]; MIF activity (8.6 mg, 57% yield) was recovered in fractions with a pI of 4.6 to 5.2. Female BALB/c mice (Jackson Labs, Bar Harbor, Me) were immunized with 160 μg of this partially purified Mo-MIF in Freund's complete adjuvant by injection into the peritoneal cavity and 3 weeks later by intravenous and intraperitoneal injection of a further 160 μg in PBS.[10]

Three days after the final injection, the spleen cells were cultured at 2.0×10^7/mL with 2.5 μg/mL MIF for three days.[11] Totals of 5×10^7 spleen cells and 2.5×10^7 NS-1 mouse myeloma cells were fused[12] and incubated at room temperature (RT) for 24 hours. Quantities of 1.2×10^5 cells per well were cultured in 96-well microtiter plates (Linbro, Flow Laboratories, McLean, Va) with 0.1 mol/L hypoxanthine, 0.4 μmol/L aminopterin and 16 μmol/L thymidine. Two weeks later, wells containing colonies were tested for anti-MIF antibody activity by incubating 50 μL of culture fluid with 0.4 μg of partially purified MIF overnight at 4 °C. Antibody-bound material was removed from the supernatants by adding 20 μL of rabbit antimouse IgG antibody bound to protein A-Sepharose (Sigma Chemical Corp, St Louis) and centrifuging.[13] The supernatants were assayed for MIF activity using an agarose microdroplet assay.[14] A colony producing an IgGl antibody that neutralized and precipitated Mo-MIF and first and second day MIF from peripheral blood lymphocytes (PBL) (data not shown) was cloned by limiting dilution and was referred to as E11. The F12, an IgG1 clone that lacks anti-MIF activity, was used as a negative control. Ascites fluid was produced in BALB/c mice primed with pristane seven days prior to injection with 5×10^6 hybrid cells. IgG concentrations were determined by a radial gel diffusion assay using rabbit antimouse IgG (Miles Scientific, Naperville, Ill).

Sodium dodecyl sulfate–polyacrylamide gel electrophosphoresis (SDS-PAGE). Samples were prepared for SDS electrophoresis[15] by heating to 100 °C for five minutes with an equal volume of 50-mmol/L Tris-HCl buffer, pH 7.5, 1% SDS, 5% mercaptoethanol, 0.005% bromophenol blue, and 10% glycerol. The following standards were used: transferrin; bovine serum albumin; rabbit IgG, heavy chain; ovalbumin; rabbit IgG, light chain; and cytochrome c–indicated mol wts of 80,000, 69,000, 52,000, 43,000, 22,000, and 12,000, respectively. The gels were stained with Coomassie blue, incubated in Enhance (New England Nuclear), dried, and subjected to fluorography.[16]

Immunoprecipitation of MIF by immobilized monoclonal anti-MIF antibody. Anti-MIF (αMIF)–MoAb bound to rabbit antimouse IgG coupled to protein A–Sepharose was prepared as follows: Seven milliters of rabbit antimouse IgG (Cappel Laboratory, Cochranville, Pa) were incubated with a 1-mL slurry of protein A–Sepharose for one hour at RT. The antimouse IgG–protein A-Sepharose was washed and mixed with 20 mL of anti-MIF MoAb at 4 °C for 12 hours and was then washed four times with RPMI 1640 containing 1% FCS, 25 mmol/L HEPES buffer, and 10 μg/mL gentamicin (anti-MIF–Sepharose). Fifty microliters of the anti-MIF–Sepharose suspension were mixed with 50 μL of the fractions to be tested and incubated for 90 minutes at 4 °C. After centrifugation the supernatants were collected for lymphokine assays.

Assay for MIF. MIF was either assayed by a capillary tube migration-inhibition assay[17] or an agarose microdroplet method.[14] In the first case, guinea pig peritoneal macrophage migration was measured from capillary tubes. In the latter case, the migration of human monocytes from an agarose droplet was determined. The human monocytes were prepared by two-step density gradient centrifugation of peripheral blood using Ficoll and Percoll gradients.[18] In both assays, one unit of MIF was defined as the amount of MIF that produced 50% inhibition of migration. To determine the number of MIF units in a given fraction, serial dilutions were assayed.

Assay for neutrophil migration inhibition. Neutrophil migration inhibition was assayed using an agarose microdroplet method[13] by determining the migration of human neutrophils from an agarose droplet. Human neutrophils were prepared by Ficoll-Hypaque density centrifugation.

Assay for CSF. CSF was assayed using a standard or micromodification of the colony-forming unit, culture (CFU-C) agar culture system.[19] Light-density, nonadherent bone marrow cells were obtained from healthy volunteers with appropriate informed consent. Cells were plated in 1-mL dishes or flat-bottomed 96-well Microtest III tissue culture plates (Falcon Labware, Oxnard, Calif) at a concentration of 2×10^5/mL. Test fractions (10 or 20 μL) were added and allowed to diffuse through the agar. Plates were incubated at 37 °C for ten to 14 days. Colonies containing >40 cells were enumerated. The quantity of 1 U/mL GM-CSF activity is the concentration necessary to obtain a stimulation of 50% of the maximal saturating CSF response.

Assay for interferon-γ (IFN-γ). Antiviral activity was assayed by assessing the inhibition of the cytopathic effect of vesicular stomatitis virus in human fibroblasts trisomic for chromosome 21, as described.[20]

Staining of individual CSF-induced colonies. Individual colonies were aspirated using a microcapillary tube. The colonies were then transferred onto a poly-L-lysine–coated glass slide, the medium removed with adsorbent paper, the slides dried rapidly, and the colonies stained with Wright-Giemsa stain. Alternatively, colonies were fixed with phosphate-buffered formalin acetone for 30 minutes at 4 °C and stained for nonspecific esterases.[21] For staining with the MoAbs anti-Mo1 (specific for monocytes, granulocytes, and null cells) and anti-My4 (specific for myeloid cells, both from Coulter Immunology, Hialeah, Fla), colonies were grown in Iscove's medium made 20% in FCS and 1.12% in methyl cellulose, incubated for 30 minutes at 4 °C with the 1:10-diluted MoAb, washed two times with PBS, and incubated with 1:20-diluted fluorescein-coupled rabbit antimouse IgG (Coulter Immunology). Mouse IgG was used as a negative control. The cells were then analyzed using an Epics 5 fluorescent-activated cell sorter (Coulter Electronics, Hialeah, Fla). Ten thousand cells were counted for each value.

RESULTS

MIF was purified from conditioned medium of the T lymphoid cell line Mo by methods previously developed for purifying MIF from circulating human lymphocytes, ie, by Sephadex G-100 gel filtration, phenyl-Sepharose affinity chromatography, and isoelectrofocusing.[1] Using the migration inhibition assay to detect and quantify MIF, 51% of the original MIF activity was recovered after the isoelectrofocusing step from fractions with a pI of 4.8 to 5.5, resulting in a 2,000-fold purification.

The protein in this partially purified fraction was labeled with tritium by reductive alkylation and subjected to reverse phase (RP)-HPLC on C-18 columns. On development with ethanol, two peaks of migration inhibitory activity were detected (Fig 1, fractions b + c and f), which separated on the basis of their slightly different hydrophobic properties. Protein was quantified by B-scintillation counting of the tritium label introduced by reductive methylation. Negligible IFN-γ activity was found in these fractions. Fractions b + c and f contained <0.01 U IFN activity/μg protein, whereas the starting fraction, conditioned medium from Mo cells, contained >700 U of IFN/μg protein. No protein could

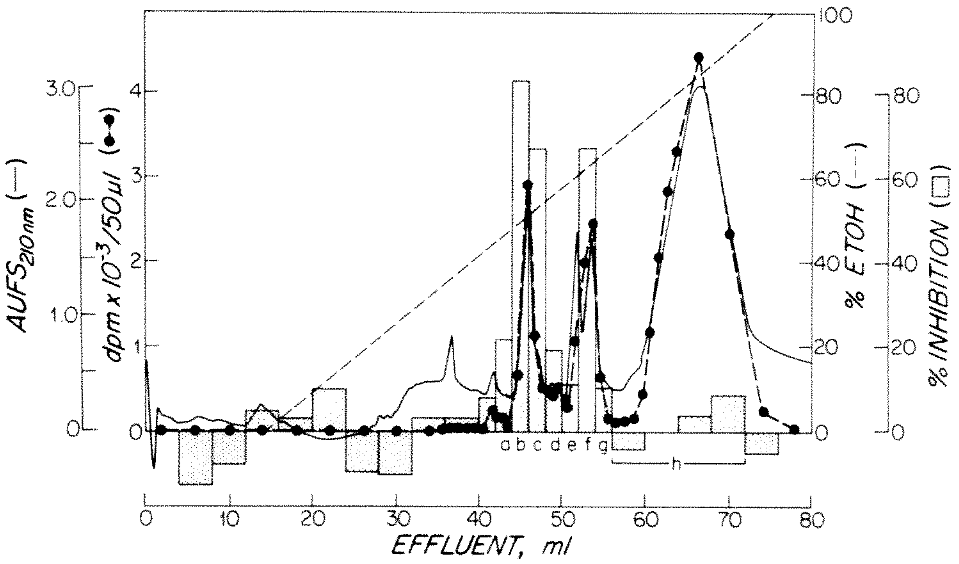

Fig 1. Fractionation of MIF produced by Mo cells using RP-HPLC. The ordinate indicates the radioactivity in 50 µL of each fraction (●---●), the extinction at 210 nmol/L (———) in absorbance units full scale (AUFS), the ethanol gradient (---), and the percent of migration of the fractions (□). The pooled active fractions of the second IEF step originating from 1.3 L of crude conditioned medium (65 µg protein) were applied to a C-18 column. One-milliliter fractions were collected into tubes containing bovine serum albumin (final concentration, 10 µg/mL). Two-milliliter fractions were pooled and dialyzed, and 30-µL portions of the pooled fractions were assayed for MIF activity. The discrepancy in the radioactivity and the extinction in fraction e + f showing one radioactive peak and two extinction peaks is based on the limited resolution of the radioactive tracing.

be detected after SDS electrophoresis by Coomassie blue or silver staining of these fractions. The purification relative to the conditioned medium of MIF in fractions b + c and f was 6,100- and 3,200-fold with a yield of 5% and 2% respectively as assessed by the determination of the radioactivity in these fractions demonstrating that a highly effective scheme for purification of MIF has been developed (Fig 2).

Analysis of fractions b + c and f by SDS electrophoresis under reducing conditions and fluorography demonstrated only one tritium-labeled protein band with 34,000 mol wt. We cannot, however, rule out the possibility that the protein band is residual PHA (see Discussion) because this band is also present in fractions that do not contain MIF activity.

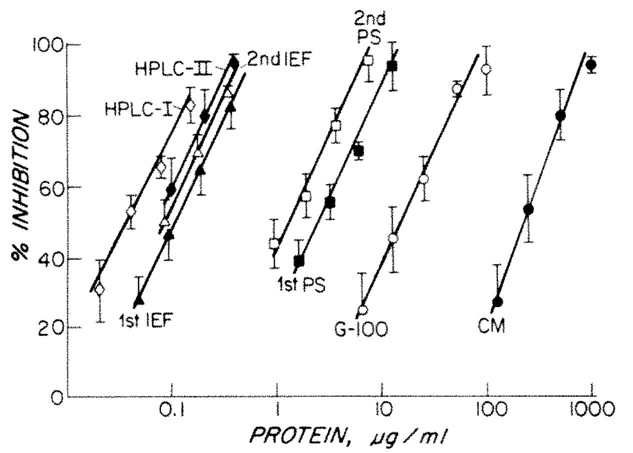

Fig 2. Specific activity of Mo-MIF after the different purification steps. The ordinate shows the MIF activity, the abscissa the protein concentration of the fractions. Abbreviations: CM, conditioned medium; G-100, Sephadex G-100 gel filtration; PS, phenyl-sepharose affinity chromatography; HPLC-I, fraction b + c from RP-HPLC; HPLC-II, fraction f from RP-HPLC.

The fractions from the RP-HPLC step were tested for CSF activity; it was found that the MIF-containing fractions b + c and f had CSF activity (Table 1). Minimal CSF activity was also detected in fraction h.

It was of interest to investigate whether the CSF activity could be absorbed by a MoAb that absorbs MIF. This antibody was specific to MIF because 100 and 500 units of IFN-γ could neither be neutralized nor precipitated by anti-MIF antibody (experiments not shown). Fractions b + c and f (8 µg of protein in 100 µL) were treated with 0.15 to 4.0 µg αMIF-MoAb bound to Sepharose and then tested for CSF activity. Table 2 shows that CSF activity is absorbed by αMIF-MoAb. This finding suggests that the MIF and CSF described are associated with the same molecule or are closely related molecules that share an identical epitope. Preliminary experiments indicate that in contrast to GM-CSF–induced colonies less than 17% of the cells from colonies induced with the MIF-associated CSF on day 14 of the culture stained with anti-Mol and less than 5% with anti-My4. These colonies consisted of tight aggregations of mononuclear esterase-negative cells lacking erythroid character. Since Mo cells produce also GM-CSF, it was of interest to find out whether GM-CSF has an effect on macrophage migration. In four experiments, GM-CSF in a concentration of 10,000 U, 1,000 U, 100 U, 10 U and 1 U/mL showed no effect in a migration inhibition assay. Whereas 1 U/mL of MIF from a Sephadex G-100 gel

Table 1. CSF Activity in RP-HPLC Fractions of Mo Cell Supernatants

Fraction* (100 μL)	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128
a	0	0	0	0	0	0	0	0
b + c	116 ± 2†	124 ± 4	102 ± 2	85 ± 2	58 ± 1	7 ± 0	31 ± 0	2 ± 1
d + e	0	0	0	0	0	0	0	0
f	150 ± 3	168 ± 1	136 ± 3	118 ± 1	89 ± 1	47 ± 2	16 ± 3	4 ± 1
g	0	0	0	0	0	0	0	0
h	38 ± 1	23 ± 11	10 ± 1	5 ± 1	1 ± 0	0	0	0

*The highest concentration is 16 ng/100 μL.

†Colony-forming units/1.8 × 10^5 cells/ml.

The data are from one typical experiment.

filtration caused 83% inhibition of migration, GM-CSF in a concentration of 10,000 to 1 U/mL inhibition of migration ranging from 5% to 13%. Furthermore, αMIF-MoAb (2.0 to 4.0 μg bound to Sepharose) did not abolish GM-CSF activity (experiments not shown). It was also of interest to investigate whether MIF from Mo cells affected the mobility of neutrophils as it was described to be the case for GM-CSF.[22] Experiments assessing human neutrophil migration inhibition using the agarose droplet migration system have demonstrated no effect of Mo-MIF/CSF on these cells (experiments not shown).

DISCUSSION

MIF from conditioned medium of the T leukemia cell line Mo (specific activity, 4.7 U/mg protein) was purified (specific activity, 2.3 × 10^4 U/mg protein) by gel filtration, phenyl-Sepharose hydrophobic chromatography, isoelectrofocusing and RP-HPLC, proving it to be a highly effective purification scheme. Two MIF species were found after fractionation on RP-HPLC. The basis of the heterogeneity of Mo-MIF is not known; minor differences in glycosylation could account for their different hydrophobic behavior. It is unlikely that the difference in hydrophobicity arose from the reductive methylation step because two active MIF species were eluted at identical positions when unlabeled MIF was fractionated by RP-HPLC (data not shown).

Table 2. Effect of αMIF-MoAb and Control MoAb on MIF and CSF Activity of RP-HPLC Fractions

	Microliters of Mab	After Treatment With Anti-MIF-MoAb	After Treatment With Control MoAb
MIF activity (% of I)	0	90 ± 5	93 ± 6
	6	65 ± 3	95 ± 2
	12	49 ± 10	96 ± 3
	25	28 ± 8	92 ± 5
	50	10 ± 7	90 ± 3
	100*	7 ± 2	91 ± 2
CSF activity (CFU-C/ 2.0 × 10^5 cells)	0	>100	>100
	4	>100	ND
	12	62 ± 10	ND
	36	12 ± 1	ND
	100	15 ± 2	>100

Abbreviation: ND, not determined.

*Containing 40 μg protein/mL. The control media in the absence of Mo-CSF activity yielded 14 ± 4 CFU-C/2 × 10^5 cells.

Data of two typical experiments are presented. The active fractions b, c, and f were pooled in the experiments.

The highly purified MIF fractions from HPLC were found to display CSF activity when tested on bone marrow cells. Furthermore, by using a MoAb selected by its ability to neutralize and to precipitate MIF, the Mo-MIF activity as well as the CSF activity associated with the MIF activity could be absorbed out. These findings indicate that MIF and CSF are either identical or are closely related and share a common structure.

It is known that the proliferation and differentiation of granulocytes and macrophage precursors is regulated by hormones collectively referred to as CSFs.[23] Although multi-CSF or interleukin 3 stimulates the production of colonies of a broad range of target cells including mast cells, neutrophils, basophils, eosinophils, megakaryocytes, erythroblasts, and macrophages,[24] other CSFs stimulate the formation of granulocytes and macrophages only (GM-CSF),[25] of macrophages (M-CSF),[26] and granulocytes (G-CSF).[27] An earlier study demonstrated that Mo cells constitutively produce a GM-CSF,[15,28,29] but this GM-CSF is functionally and biochemically distinct from the lymphokine CSF associated with the MIF described in this communication. Recent studies have shown that purified and biosynthetic (recombinant) GM-CSF, in contrast to MIF-CSF, is able to stimulate colony formation by KG1 cells and is identical to a lymphokine acting on mature neutrophils referred to as NIF-T.[22,30] In addition, we could show that GM-CSF does not affect monocyte migration and Mo-MIF CSF does not affect neutrophil migration. We do not yet know the specificity of MIF CSF or the range of hematopoietic colonies directly stimulated by this mediator. A detailed analysis of colony types stimulated directly, or indirectly via accessory cells, will be undertaken when adequate homogeneous material is available.

Although the functional role of the MIF/CSF is not known, we assume that it plays a crucial role in the activation of mononuclear phagocytes and expansion of effector cells in host defense. It may also be important in localized immunologic events such as granuloma formation. The concept that lymphokines acting on mature granulocytes and mononuclear phagocytes also stimulate their production has been supported by studies in the mouse with L cell CSF[31,32] and

GM-CSF.[33] Also, partially purified human placental CSF is able to stimulate neutrophil antibody–dependent cell-mediated cytotoxicity.[34] Our findings indicate the molecular relatedness of human MIF and a species of CSF produced by activated T lymphocytes.

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REFERENCES

1. Weiser WY, Greineder DK, Remold HG, David JR: Studies on human migration inhibitory factor: Characterization of three molecular species. J Immunol 126:1958, 1981

2. Remold HG, Mednis AD, Kawaguchi T, Bersch N, Golde DW: Production of migration inhibitory factor by a human T-lymphoblast cell line. Cell Immunol 78:305, 1983

3. Saxon A, Stevens RH, Quan SG, Golde DW: Immunological characterization of hairy cell leukemias in continuous culture. J Immunol 120:777, 1978

4. Nathan I, Groopman JR, Quan SG, Bersch N, Golde DW: Immune (gamma) interferon produced by a human T-lymphoblast cell line. Nature 292:842, 1981

5. Iscove NN, Melchers F: Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. J Exp Med 147:923, 1978

6. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Clark SC, Wang EA: Molecular cloning of human and Gibbon T-cell–derived GM-CSF cDNAs and purification of the natural and recombinant human proteins. Cancer Cells 3: Growth Factors and Transformation. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1985, p 235

7. Remold HG, Mednis AD: Two migration inhibitory factors with different chromatographic behavior and isoelectric points. J Immunol 118:2015, 1977

8. Lowry OH, Rosebough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265, 1951

9. Jentoft N, Dearborn DG: Labeling of proteins by reductive methylation using sodium cyanoborohydride. J Biol Chem 254:4359, 1979

10. Weiser WY, Remold HG, David JR: Generation of human hybridomas producing migration inhibitory factor (MIF) and of murine hybridomas secreting monoclonal antibodies to human MIF. Cell Immunol 90:167, 1985

11. Fox PC, Berenstein EH, Siraganian RP: Enhancing the frequency of antigen-specific hybridomas. Eur J Immunol 11:431, 1981

12. Gefter ML, Margulies DH, Scharff MD: A simple method for polyethylene glycol–promoted hybridization of mouse myeloma cells. Somatic Cell Genet 3:231, 1977

13. Lampson LA: Immunoprecipitation with monoclonal antibodies, in Kennet RH, McKearn TJ, Bechtol KB (eds): Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analysis. New York, Plenum, 1980, p 395

14. Harrington JT, Stastny P: Macrophage migration from an agarose droplet: Development of a micromethod for assay of delayed hypersensitivy. J Immunol 110:752, 1973

15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

16. Bonner WM, Laskey RA: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46:83, 1974

17. David JR, David RA: Assay for inhibition of macrophage migration, in Bloom BR, Glade PR (eds): In Vitro Methods in Cell-mediated Immunity. Orlando, Fla, Academic, 1971, p 249

18. Vadas MA, David JR, Butterworth A, Sherry B, Dessein A, Hogan M, Bout D, David J: Interactions between human eosinophils and schistosomula of schistosoma mansoni I stable and irreversible antibody-dependent adherence. J Immunol 124:1441, 1979

19. Golde DW, Quan SG, Cline MJ: Human T lymphocyte cell line producing colony-stimulating activity. Blood 52:1068, 1978

20. Weiser WY, Remold HG, Block LH, David JR: Dissociation of human interferon gamma like activity from migration inhibition factor. Cell Immunol 88:109, 1984

21. Kaplow LS: Cytochemical identification of mononuclear macrophages, in Herscowitz HB, Holden HT, Bellanti JA, Ghaffar A (eds): Manual of Macrophage Methodology. New York, Marcel Dekker, 1981, p 199

22. Gasson YC, Weisbart RH, Kaufman SE, Clark S, Hewick RM, Wong GG, Golde DW: Purified human granulocyte-macrophage colony stimulating factor: Direct action on neutrophils. Science 226:1339, 1984

23. Nicola NA, Vadas M: Hemopoietic colony-stimulating factors. Immunol Today 5:76, 1984

24. Metcalf D: Regulation of self-replication in normal and leukemic stem cells, in Golde DW, Marks PA (eds): Normal and Neoplastic Haematopoiesis. New York, Liss, 1983, p 141

25. Burgess AW, Camakaris J, Metcalf D: Purification and properties of colony-stimulating factor from mouse lung–conditioned medium. J Biol Chem 252:1998, 1977

26. Stanley ER, Heard PM: Factors regulating macrophage production and growth. Purification and some properties of the colony-stimulating factor from medium conditioned by mouse L cells. J Biol Chem 252:4305, 1977

27. Nicola NA, Metcalf D, Matsumoto M, Johnson GR: Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. J Biol Chem 258:9017, 1983

28. Lusis AJ, Quon DH, Golde DW: Purification and characterization of a human T-lymphocyte–derived granulocyte-macrophage colony-stimulating factor. Blood 57:13, 1981

29. Lusis AJ, Golde DW, Quon DH, Lasky LA: Translation of m RNA for human granulocyte-macrophage colony stimulating factor. Nature 298:75, 1982

30. Weisbart RH, Lusis AJ, Chan G, Billing R, Ashman RF, Golde DW: Neutrophil migration inhibition factor from T lymphocytes (NIF-T): Selective removal of biological activity by human peripheral blood neutrophils, myelocytic leukemia cells, and differentiated HL-60 cells. J Immunol 128:457, 1982

31. Wing EJ, Waheed A, Shadduck RK, Nagle LS, Stephenson K: Effect of colony-stimulating factor on murine macrophages. Induction of antitumor activity. J Clin Invest 69:270, 1982

32. Tushinski RJ, Stanley ER: The regulation of macrophage protein turnover by a colony-stimulating factor (CSF-1) J Cell Physiol 116:67, 1983

33. Lopez A, Nicola N, Burgess AW, Metcalf D, Battye FL, Sewell W, Vadas M: Activation of granulocyte cytotoxic function by purified mouse colony-stimulating factors. J Immunol 131:2983, 1983

34. Vadas MA, Nicola N, Metcalf D: Activation of antibody-dependent cell-mediated cytotoxicity of human neutrophils and eosinophils by separate colony-stimulating factors. J Immunol 130:795, 1983

Altered Oxidative Product Formation in Neutrophils of Patients Recovering From Therapy for Acute Leukemia

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During chemotherapy for acute leukemias, severe neutropenia allows acquisition of life-threatening infections that are difficult to clear with antibiotics alone. With return of myelopoiesis, even severe infections often improve dramatically. We have sequentially examined oxidative metabolic responses of polymorphonuclear leukocytes (PMNL) from 30 patients with acute leukemias before induction chemotherapy and after recovery of myelopoiesis (circulating PMNL >500/μL). Maximal oxidative metabolic responses were quantitated by flow cytometric analysis of H_2O_2-dependent oxidation of intracellular 2′,7′-dichlorofluorescin (DCFH) in individual PMNL after stimulation with phorbol myristate acetate (PMA). Resting PMNL oxidized a mean of 6.8 attomoles (amol) DCFH/cell/15 min, with no difference between normal or patients' PMNL. PMA-stimulated normal PMNL oxidized 183 ± 35 amol/cell (mean ± SD, n = 120). In patients' PMNL obtained before chemotherapy, the mean DCFH oxidation was not significantly different from controls (216 ± 78 amol/cell). However, 11 of 22 samples revealed populations of granulocytes with increased (primed) oxidative responses; seven of these 11 patients had proven or suspected infection at presentation. At recovery from chemotherapy-induced neutropenia, PMNL from 19 of 21 patients possessed one or more significant subpopulations with primed oxidation in response to PMA. In these 19 patients, 61% ± 8% of PMNL comprised primed populations that oxidized 503 ± 46 amol/cell. Oxidative activity was most pronounced in patients with proven or clinically suspected infections (with 41% ± 9% of PMNL oxidizing 615 ± 79 amol/cell). However, oxidative responses to PMA were also significantly increased in recovery PMNL from ten patients without clinical or laboratory evidence of active infection (79% ± 11% of PMNL primed to oxidize 402 ± 29 amol/cell). The peak responses of the primed subpopulations were short-lived and generally lasted three days or less, although oxidative responses remained elevated above normal for a week or more. All of the patients with increased PMNL responsiveness survived their hospitalization. In contrast, PMNL from four patients had a significant population (18% to 82% of cells) with reduced responsiveness. Two of these four patients (with 71% and 75% subnormal cells) died during this induction attempt; the third died during a second induction attempt; only one survived to discharge. The clinical significance of these phenomena is yet to be determined. The results support the clinical observation that recovery after therapy for acute leukemia suggests the presence of granulocytes with potent functional activity. Priming apparently may occur in the absence of overt infection, possibly because of stimulatory substances released during the period of prolonged neutropenia. Only a small number of patients were observed with the opposite phenomenon of hypoactive PMNL; however, the high mortality in this group suggests that the normal response (and/or the ability to prime PMNL following neutropenia) may be important to the eventual clinical outcome.

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ACUTE INFECTIONS are the predominant cause of death and the major source of morbidity in patients with acute leukemia.[1-3] Induction chemotherapy is usually characterized by 3 or more weeks of profound granulocytopenia. In almost all patients this period is complicated by fevers, with or without culture-documented infections; an infectious cause is identified in more than half the febrile episodes.[1] Patients are at greatest risk during this period of severe neutropenia.[1,4] Dramatic clinical improvement, including resolution of fevers and infections, is frequently observed with the reappearance of circulating polymorphonuclear leukocytes (PMNL), even in very ill patients. However, Bodey et al found that even patients with >1,500 PMNL/μL spent 6% to 23% of hospital days with infections.[4] It is unknown whether the frequent improvement and occasional worsening of clinical states might be associated with increased or decreased functional capabilities of the PMNL appearing in the blood at the time of recovery from chemotherapy (recovery PMNL).

Prior reports of granulocyte characteristics and function in patients with acute leukemia have described varying abnormalities in adherence,[5] in vivo migration,[6,7] in vitro chemotaxis,[8,9] granule enzyme content,[10-14] phagocytosis,[5,8,15-18] oxidative metabolism,[19-21] and bactericidal[13,16,18,22-25] and candidacidal[9,13,26-28] activities. Other investigators have found normal in vitro functions,[29-31] and some of the aforementioned reports have revealed one or more normal functional activities in the presence of specific functional defects.[18,22,25,26,28] Several of these studies have evaluated patients in remission as well as with active disease; most have shown resolution or improvement of the abnormalities during remission,[6,8,10-12,14,18,22,27] but persistent defects have been demonstrated.[21,25] Among studies focusing only on remission patients, both normal[29,30] and abnormal[9,23] PMNL function has been described. Functions of PMNL obtained immediately postrecovery from chemotherapy have not been specifically examined.

Previous studies of PMNL function have generally been limited by assay techniques that measure mean activity of bulk cell populations, frequently an admixture of mature and immature granulocytes. For example, a 50% mean decrease in functional activity of a group of cells may reflect zero activity in half of the cells and normal activity in half, a 50%

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decrease in activity of all cells, zero activity in 75% of cells and twice normal in 25% of cells, or any of an infinite number of such combinations. Granulocyte heterogeneity, even among mature PMNL, is now well recognized in the healthy as well as the diseased host.[5,32,33] We used a technique that has successfully identified heterogenous subpopulations of PMNL in nonleukemic patients with acute bacterial infections.[33] An assay for hydrogen peroxide (H_2O_2) in aqueous solutions[34] was modified to quantitate H_2O_2-dependent oxidative product formation by flow cytometry.[35,36] Flow cytometry enabled us to identify and assay only mature granulocytes and to measure metabolic activity of single PMNL obtained from blood with as few as 500 PMNL/μL. Therefore, PMNL subpopulations could be identified and characterized even when the absolute granulocyte count was low or immature granulocytes were present in the peripheral blood. This technique was used to define the oxidative potential of PMNL in patients with acute leukemia before and after remission induction.

MATERIALS AND METHODS

Patient selection and clinical evaluation. Between May and Nov 1984 all patients at the Bowman Gray School of Medicine receiving induction chemotherapy for acute leukemia (lymphocytic [ALL], nonlymphocytic [ANLL], chronic granulocytic leukemia in blast crisis [CGL-BC]) were asked to participate in a study of PMNL functions. In patients who gave written informed consent, blood samples were obtained prior to initiation of therapy when feasible and at least thrice weekly, often daily, from the time of initial WBC recovery ($\geq 500/\mu$L) until normalization of results or discharge. Initially, additional samples were obtained three times weekly even during the period of leukopenia; however, this practice was discontinued since a prohibitive amount of blood would have been required to obtain an adequate number of PMNL for study. Studies became feasible when circulating PMNL were $\geq 500/\mu$L. The patients were examined daily with special attention to clinical and laboratory evidence of infection. Patients were classified as having proven (clinical observations and positive culture or chest x-ray), suspected (fever and/or physical findings without culture positivity), or no evidence of infection. Control PMNL were obtained from noninfected normal human volunteers.

PMNL isolation and assay techniques. Heparinized whole blood samples were obtained from indwelling central venous catheters or by standard venipuncture techniques. Isolation of PMNL by sedimentation, centrifugation through Ficoll/metrizoate, hypotonic lysis of erythrocytes, and the 2',7'-dichlorofluorescin (DCFH) oxidation assay were used as described previously.[35] In brief, isolated PMNL (10^6/mL) were incubated for 15 minutes at 37 °C in 5 μmol/L dichlorofluorescin diacetate (Eastman Kodak, Rochester, NY) in phosphate-buffered saline containing 0.1% gelatin, without Ca^{++} or Mg^{++}. Dichlorofluorescin diacetate readily diffuses into cells, is hydrolyzed to 2',7'-dichlorofluorescin (DCFH), a nonfluorescent fluorescein analogue, and is trapped within the cells. During the oxidative metabolic burst of PMNL, DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF). Baseline fluorescence was measured on a Spectrum III or a Cytofluorograph 50H flow cytometer equipped with a 4-W argon laser (488-nm emission, 250-mW output) and a 2150 computer (all from Ortho Diagnostic Systems, Westwood, Mass). Immediately before addition of stimulant, EDTA (2 mmol/L final concentration) was added to prevent cellular aggregation. PMNL were then stimulated with phorbol myristate acetate (PMA) (100 ng/mL). Fluorescence of individual resting and PMA-stimulated PMNL was measured after 15 minutes of incubation with horizontal agitation in a Dubnoff water bath. Mature PMNL were distinguished from immature granulocytic precursors using a combination of low-angle, forward- and right angle-scattered laser light (Fig 1). Individual PMNL fluorescent emission (510 to 550 nm) was determined by computer gating with calibration to measure the fluorescent intensity in attomoles (1 amol = 10^{-18} mol) DCF per cell, as described.[35]

The intracellular oxidation of nonfluorescent DCFH to fluorescent DCF is mediated by H_2O_2 generated by the PMNL oxidative burst. This reaction occurs within the cytosol[35] and is in competition with other cytosolic H_2O_2-consuming activities including myeloperoxidase-mediated oxidation, lipid peroxidation, and H_2O_2 destruction by catalase or the glutathione system. Therefore, increased DCF formation could reflect a decrease in these competing cytosolic reactions. To evaluate this possibility, PMNL from patients and controls were also evaluated in a similar fashion following the addition of an extracellular oxidant-generating system, xanthine oxidase (30 mU/mL) plus 10 mmol/L acetaldehyde, as described.[33] We have noted significant variability among different batches of commercially available xanthine oxidase; therefore, the xanthine oxidase data are expressed as the ratios of DCF fluorescence generated by 15 minutes' exposure to xanthine oxidase for patient's PMNL divided by that for parallel control PMNL.

Oxidative activity of each patient's PMNL population was displayed in a histogram as seen in Fig 2. A mean for the total population and for each subpopulation was then calculated from the histogram. Subpopulations were defined as the mean and proportion of cells above and below the nadir of cell distribution between the two populations present. Subpopulations were generally classified as increased (primed), normal, or decreased (below normal) in comparison with the response of parallel control cells.

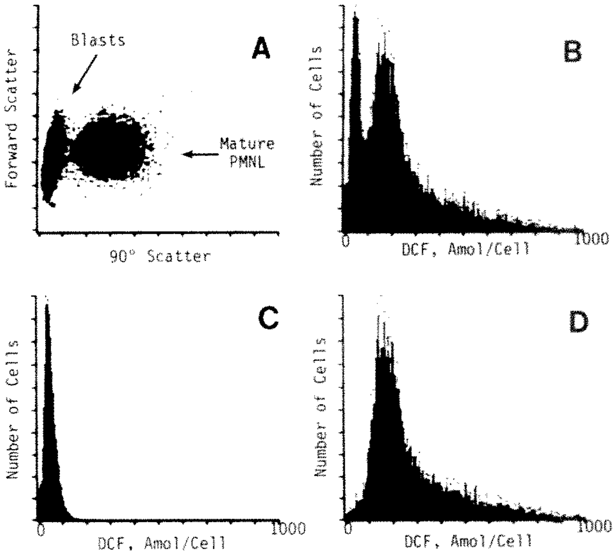

Fig 1. Analysis of the DCF content of isolated (through Ficoll/metrizoate) PMNL from a patient with ANLL. The total cells present would appear as in (A). From the characteristics of (B) scattered laser light, the population of (C) blasts or (D) mature PMNL can readily be distinguished and selected by the computer for fluorescence analysis, shown in the lower left and lower right, respectively. Analysis was performed on the Cytofluorograph 50H.

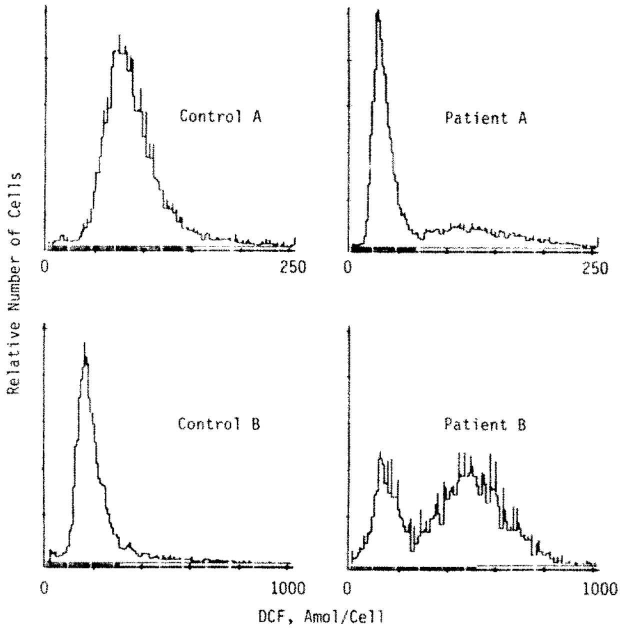

Fig 2. Representative histograms of DCF responses of leukemic patients' PMNL after recovery from induction chemotherapy. Note the different scales on the horizontal (DCF) axes between the upper and lower histogram pairs. The upper right is a patient with a large defective population of PMNL; the lower right is a patient whose PMNL are a mixture of normal and primed cells. Parallel controls for each patient are shown on the left. In each, PMNL were stimulated for 15 minutes with PMA. These data were obtained using the Spectrum III flow cytometer.

RESULTS

Clinical characteristics of the patients. Thirty adults with acute leukemia (25 ANLL, 4 ALL, 1 CGL-BC) participated in this study; one patient elected not to participate. Nineteen patients met the criteria for evaluation including PMNL recovery. Eleven patients failed to complete the study; nine (eight ANLL, one CGL-BC) died during induction, and two patients (one ANLL, one ALL) were discharged prior to PMNL recovery. Two patients with ANLL completed two separate inductions for a total of 21 evaluable induction cycles with granulocyte recovery.

Studies of PMNL prechemotherapy. Pretreatment samples were obtained from 22 of 30 patients (Table 1) and from 14 of 19 patients who subsequently had recovery samples evaluated. Eight of the 22 patients with pretreatment samples had proven infection prior to therapy; seven had suspected and seven had no evidence of infection. In the DCFH oxidation assay, normal PMA-stimulated PMNL oxidized 183 ± 35 amol DCFH/cell/15 min (mean ± SD, N = 120); the normal range was therefore defined as 113 to 253 amol/cell/15 min. The oxidative responses of pretreatment PMNL were variable and, on the average, oxidized 213 ± 77 amol DCFH/cell (range, 101 to 369), but 11 patients had granulocytes with oxidative activity increased above the normal range. In three patients, the oxidative population distribution was unimodal, with a modest increase (range, 265 to 279 amole/cell) in oxidative activity of all PMNL present. Eight patients, seven with suspected or proven infections, had distinct subpopulations with primed oxidative activity in response to PMA stimulation. A mean of 39% (range, 18% to 75%) of PMNL exhibited priming in these eight patients with a mean oxidation of 423 (range, 330 to 573) amol DCFH per cell in the primed population; six of eight of these patients survived their induction hospitalization.

Nine patients possessed 10% to 100% (mean 43%) of PMNL with a subnormal response to PMA (less than 113 amol/cell), with a mean DCF intensity of 66 amol (range, 36 to 106). Eight of these nine patients had suspected or proven infections at presentation, and only three of the nine survived their hospitalization. The three who survived all had a cell distribution that also included a primed subpopulation as previously described. Among patients who survived their hospitalization, no correlation between pretreatment oxidative response to PMA and response to chemotherapy was identifiable.

Studies of PMNL at recovery from chemotherapy. Table 2 displays patient characteristics, clinical data, and mean and subpopulation distributions of DCF intensities of PMA-stimulated recovery PMNL from the 19 patients who completed the study. The DCF data are summarized in Fig 3.

In 11 of 21 inductions clinically proven (nine) or suspected (two), infections were present at the time of recovery. The mean DCFH oxidized per cell by parallel control PMNL was 176 ± 29 amol/15 min (mean ± SD) after PMA stimulation with a unimodal distribution. The mean oxidative response to PMA stimulation in PMNL from recovering patients was elevated to 340 ± 138 amol/cell/15 min, and more importantly the distribution of cells was frequently not unimodal. Thirteen of 21 patients had two or three PMNL subpopulations, and 20 of 21 possessed populations with increased DCFH oxidation (1.5 to 6 times control). In these patients a mean of 58% (range, 9% to 100%) of the cells demonstrated priming. No clear correlations were noted between the percentage or degree of priming of PMNL and the type of leukemia or the presence of infection. However, a unimodal distribution appeared to be more common in noninfected patients, and the most dramatic oxidative bursts did occur in patients with proven severe infections (eg, patients 1 and 2). The peak oxidative activity was short-lived, often lasting only one to three days. However, PMNL continued to show priming for a week or longer (Fig 4).

Subnormal oxidative activity was observed in a significant proportion (>10%) of PMNL from four patients whose leukemia was not completely eradicated by their induction chemotherapy. Two of these patients subsequently died from overwhelming infections during their hospitalization; in each of these, the defective PMNL constituted a majority of the PMNL population, and little or no successful priming of circulating PMNL occurred. In both these patients the defective responses were observed on two or more occasions and persisted more than 1 week. The other patients with defective cells recovered from the induction regimen; however, one of these died after a subsequent induction attempt. Only one of the patients with defective PMNL responses was

Table 1. Oxidative Responses of PMNL Obtained Before Chemotherapy, Grouped by Subsequent Outcome

				DCF in Attomoles/Cell (Percentage of total PMNL)*				
Response	Age/Sex	Diagnosis	Infected at Admission	Control	Mean	Low Pop	Normal Pop	Primed Pop
Survived and obtained complete remission	65/M	ANLL—New	P	165	369		249 (63)	573 (37)
	56/F	ANLL—New	P	209	273		231 (82)	461 (18)
	47/M	ANLL—RD	S	184	155	95 (75)		330 (25)
	36/F	ANLL—RD	S	164	221	49 (26)	203 (38)	333 (37)
	58/F	ANLL—RD	S	152	156		156 (100)	
	35/F	ALL—New	N	158	279			279 (100)
	60/M	ANLL—New	N	191	248		248 (100)	
Survived with persistent leukemia or partial remission	68/M	ANLL—New†	P	192	131	44 (35)	178 (65)	
	63/F	ANLL—RD	P	153	168		168 (100)	
	42/M	ALL—RD	S	152	291	49 (14)	199 (56)	515 (31)
	68/M	ANLL—RD	N	193	265		265 (100)	
	68/F	ALL—RD	N	158	136		121 (73)	364 (26)
	71/M	ANLL—New	N	191	268		268 (100)	
Died after quantitative granulocyte recovery	60/F	ANLL—New	P	158	101	56 (71)	237 (29)	
	47/F	ANLL—New	N	158	222		222 (100)	
Died during period of granulocytopenia	73/F	ANLL—New	P	187	341		130 (25)	409 (75)
	43/F	ANLL—New	P	162	314		154 (33)	401 (66)
	65/M	ANLL—RD	P	181	163	63 (17)	182 (83)	
	15/M	ANLL—New	S	182	129		129 (100)	
	52/M	CGL—BC	S	198	211	94 (43)	237 (57)	
	68/M	ANLL—New	S	200	106	106 (100)		
	46/M	ANLL—RD	N	231	197	36 (10)	214 (90)	

Abbreviations: RD, relapsed or refractory disease; P, proven; S, suspected; N, none.

*DCF content of PMNL, 15 minutes after stimulation with PMA. Low population (POP) was defined as populations with a mean DCF response below the normal range (<113 amol DCF/cell). Primed populations had a mean above the normal range (>253 amol/cell). Normal populations had mean DCF between 113 and 253 amol/cell. The percentage of total mature PMNL in each population is shown in parenthesis.

†This patient died after a subsequent induction attempt.

discharged from the hospital. No patients who cleared their leukemia (CR) had subpopulations with defective oxidation.

Studies regarding altered efficiency of patients' cells in the DCFH oxidation assay. Oxidation of DCFH by xanthine oxidase/acetaldehyde, an extracellular oxidant, was unimodal in subjects and controls alike. This suggests uniformity in the efficiency of DCF oxidation within the cytosol. In contrast, bimodal (and in two patients trimodal) responses of patients' PMNL after PMA stimulation suggest that the subpopulation responses were due to differential endogenous oxidant generation by the respiratory burst. The amount of DCFH oxidized by xanthine oxidase did vary; however, there was no significant correlation between the effect of xanthine oxidase (to monitor assay efficiency in patients' cells) and endogenous oxidation after PMA stimulation ($r = .23$, $N = 21$). Normalized patient DCF were calculated using the product of the raw DCF and the ratio of control/patient response of xanthine oxidase. Although the absolute DCF values were changed by this calculation, patterns of response and the overall results were the same (results not shown).

DISCUSSION

The results of this investigation support a time-proven clinical observation—recovery PMNL possess potent functional activity. Understanding of granulocyte function has been limited by bulk cell analyses in prior studies. The flow cytometric quantitation of H_2O_2-dependent DCFH oxidation provided several advantages over standard assays that measure mean oxidative metabolic responses of bulk cell populations. Scattered laser light could readily distinguish mature PMNL from blasts, allowing accurate analysis of only the mature circulating PMNL. Small numbers of cells were required; accurate studies could be obtained with 20 mL of blood from patients with fewer than 500 PMNL/μL. Subpopulations of PMNL with reduced (deactivated) or increased (primed) responsiveness could be detected, and the specific metabolic activity of each subpopulation was quantitated.

Pretreatment studies were notable for two important findings. First, eight of 22 patients, seven of whom had proven or suspected infections, had a subpopulation of primed PMNL. This pattern of response is similar to that described in earlier studies of infected nonleukemic patients[33] and provides supportive evidence for the presence of a population of granulocytes with normal oxidative function and with the ability to respond appropriately to infection compared with nonleukemic patients. In contrast, a subpopulation of relatively inactive cells was also identified in nine patients. In three of the patients the deactivated PMNL population was relatively large (range, 26% to 71% of cells) and the oxidative responses markedly below normal (44 to 56 amol/cell/15 min). An equivalent population has not been

Table 2. Clinical Characteristics and DCFH Oxidation of PMNL Obtained at Time of Granulocyte Recovery

						Infection		DCFH, amol/Cell (Percentage of total PMNL)*			
Outcome	Patient	Age/Sex	Diagnosis	Therapy†	Response‡	During Therapy	At Recovery	Mean	Low Pop	Mid Pop	Upper Pop
Survived and obtained CR	1	47/M	ANLL	LoDAC	CR	P	P	575		401 (69)	954 (31)
	2	53/M	ANLL	HiDAC/ASP	CR	P	P	563		292 (54)	881 (46)
	3§	42/M	ALL	HiDAC/ASP	CR	P	P	377		258 (67)	613 (33)
	4	36/M	ANLL	AraC/DNR	CR	P	P	347		196 (60)	574 (40)
	5	58/F	ANLL	DHAD	CR	P	P	438			438 (100)
	6	56/F	ANLL	HiDAC/ASP	CR	P	P	266			266 (100)
	7	49/F	ANLL	HiDAC/ASP	CR	P	N	292		168 (67)	548 (33)
	8	65/M	ANLL	LoDAC	CR	P	N	258		184 (75)	497 (25)
	9	35/F	ALL	VCR/DNR/ASP/PRED	CR	P	N	446			446 (100)
	10	60/M	ANLL	HiDAC/ASP	CR	P	N	407			407 (100)
	11	36/F	ANLL	AraC/DNR	CR	P	N	379			379 (100)
	12§	63/F	ANLL	AraC/DNR	CR		N	319			319 (100)
Survived with PR or persistent leukemia	13	65/M	ANLL	AraC/DNR	PL	P	P	173		127 (81)	366 (19)
	14§	63/F	ANLL	HiDAC/ASP	PL	P	P	139	101 (82)		335 (18)
	15§	42/M	ALL	VCR/DNR/ASP/PRED	PR	P	S	341	42 (5)	275 (68)	561 (27)
	16	68/M	ANLL	HiDAC/ASP	PR	P	S	594		303 (43)	815 (57)
	17	68/F	ALL	AraC/VP16	PL	S	N	315		245 (60)	420 (40)
	18	54/M	ANLL	AMSA	PL	S	N	308			308 (100)
	19‖	68/M	ANLL	AraC/DNR	PL	P	N	371	100 (18)		432 (87)
Died after quantitative granulocyte recovery	20	47/F	ANLL	HiDAC/ASP	ID	P	P	133	69 (71)	187 (21)	482 (9)
	21	60/F	ANLL	HiDAC/ASP	ID	P	N	102	67 (75)	205 (25)	

*DCF responses, PMNL populations, diagnosis, and presence of infection, defined as in Table 1.

†Therapy: HiDAC, high-dose cytosine arabinoside; ASP, L-asparaginase; AraC, standard cytosine arabinoside; DNR, daunorubicin; VCR, vincristine; PRED, prednisone; DHAD, mitoxantraone; LoDAC, low-dose cytosine arabinoside; MTX, methotrexate; AMSA, amsacrine.

‡Responses: CR, complete remission; PL, persistent leukemia; PR, partial remission; ID, induction death.

§Patients who were evaluated during two separate inductions.

‖Died after subsequent induction attempt.

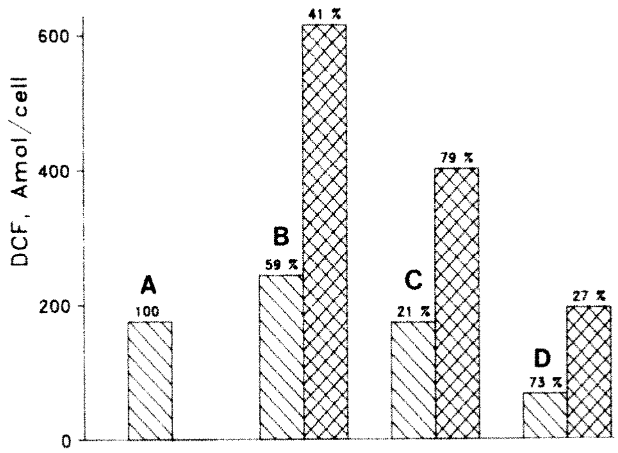

Fig 3. Summary of DCFH oxidation by stimulated PMNL obtained from patients at time of recovery from chemotherapy-induced neutropenia. Lower and upper populations were defined as those PMNL with less or more DCF than the nadir of the distribution when two populations were present. The bars represent the mean DCF in each population. The numbers above the bars are the mean percentage of PMNL in each (lower or upper) population. Patients were divided by status at time of study (A) Controls; (B) Infected, lived; (C) Not infected, lived; (D) Died. □ Lower PMNL pop; □ Upper PMNL pop.

identified in either control cells or PMNL from nonleukemic patients with infections. This might suggest that these granulocytes either originate from a leukemic clone[6,8,10,12,26,27] or are depressed by a leukemia-related inhibitor of PMNL function.[8,26-28] In further support of this concept, five of nine patients who failed to obtain a complete remission had a depressed PMNL population at recovery from chemotherapy. Such a depressed PMNL population was not identified in any patient who achieved complete remission.

Responses to PMA stimulation in recovery granulocytes were unique and consistent. All patients but one had a population of PMNL with increased DCFH oxidation. This population was evident independently of the presence, type or site of infection, type of leukemia, type of therapy, or response to therapy. However, the most dramatic oxidative responses did occur in patients with severe proven infection.

The mechanism(s) of priming of PMNL have not been defined. The presence of distinct PMNL subpopulations appears to be unique to situations of leukopoietic stress as demonstrated in prior studies of patients with bacterial infections[33] and the current data on patients after chemotherapy for acute leukemia. The stimulant in the recovering leukemia patients, especially the noninfected patients, is unknown. Perhaps clinical or subclinical infections provided the stimulus. All patients did have evidence of infection at some period during their hospitalization, but ten of 21 were

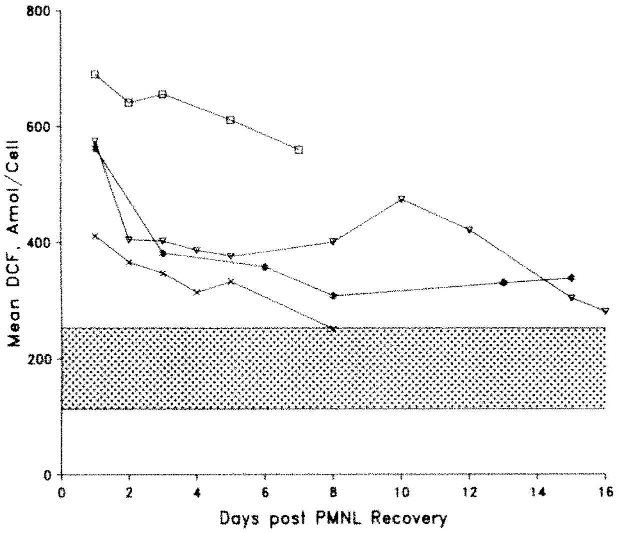

Fig 4. Time course of the mean responses of PMNL from four representative patients with acute leukemia studied after recovery from chemotherapy-induced neutropenia. Day 1 is the first day the circulating PMNL rose above 500/μL.

free of evidence of infection at the time of granulocyte recovery. As previously noted, similar primed PMNL subpopulations have been observed in bacteremic nonleukemia patients[33]; however, the degree of PMA-stimulated DCFH oxidation, even in severely infected patients, has generally been less than that seen with recovery in patients with acute leukemia. It is also possible that a leukemia-associated inhibitor of PMNL maturation and/or function is eliminated by therapy and therefore allows for full functional activity in recovering normal PMNL; this seems unlikely since very active populations were noted in some patients (eg, no. 16) with incomplete eradication of leukemia cells. A more likely possibility is a granulocyte-stimulating factor produced during the prolonged and profound granulocytopenia. One is tempted to surmise that the duration and/or degree of granulocytopenia plays a major role in the priming phenomena and may be further potentiated by infections.

The significance of these primed recovery PMNL is difficult to assert at present. The importance of their role is suggested, though by no means proven, by the infectious deaths of both patients 20 and 21 who had a definite paucity of primed cells and a predominance of relatively inactive PMNL. Although the clinical correlates, and indeed the relevance, of the primed PMNL oxidative response are yet to be determined, these and future studies using single-cell techniques should help to elucidate the functional characteristics of pretreatment and recovery granulocytes in patients with acute leukemia.

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REFERENCES

1. Bodey GP, Rodriguez V, Chang H-Y, Narboni G: Fever and infection in leukemic patients. A study of 494 consecutive patients. Cancer 41:1610, 1978

2. Estey EH, Keating MJ, McCredie KB, Bodey GP, Freireich EJ: Causes of initial remission induction failure in acute myelogenous leukemia. Blood 60:309, 1982

3. Bodey GP, Bolivar R, Fainstein V: Infectious complications in leukemic patients. Semin Hematol 19:193, 1982

4. Bodey GP, Buckley M, Sathe YS, Freireich EJ: Quantitative relationships between circulating leukocytes and infection in patients with acute leukemia. Ann Intern Med 64:328, 1966

5. Penny R, Galton DAG: Studies on neutrophil function. II. Pathological aspects. Br J Haematol 12:633, 1966

6. Holland JF, Senn H, Banerjee T: Quantitative studies of localized leukocyte mobilization in acute leukemia. Blood 37:499, 1971

7. Perillie PE, Finch SC: Quantitative studies of the local exudative cellular reaction in acute leukemia. J Clin Invest 43:425, 1964

8. Coiffier B, Frobert Y, Revol L: Polymorphonuclear function in acute myeloblastic leukemia. Biomedicine 27:94, 1977

9. Reid MM, Craft AW, Low WT: Neutrophil function in children with acute lymphoblastic leukaemia in the presence and absence of viral infections. Arch Dis Child 54:619, 1979

10. Suda T, Onai T, Maekawa T: Studies on abnormal polymorphonuclear neutrophils in acute myelogenous leukemia: Clinical significance and changes after chemotherapy. Am J Hematol 15:45, 1983

11. Schofield KP, Stone PCW, Stuart J: Quantitative cytochemistry of blood neutrophils in acute myeloid leukaemia. Br J Haematol 54:261, 1983

12. Odeberg H, Olofsson T, Olsson I: Primary and secondary granule contents and bactericidal capability of neutrophils in acute leukemia. Blood Cells 2:543, 1976

13. Pinkerton PH, Robinson JB: Granulocyte function in untreated acute and chronic granulocytic leukemia. Acta Haematol (Basel) 56:65, 1976

14. Bendix-Hansen K, Nielsen HK: Myeloperoxidase-deficient polymorphonuclear leukocytes. (II) Longitudinal study in acute myeloid leukaemia, untreated, in remission and in relapse. Scand J Haematol 31:5, 1983

15. Tornyos K: Phagocytic activity of cells of the inflammatory exudate in human leukemia. Cancer Res 27:1756, 1967

16. Yamamoto K, Sasada M, Nishiyama H, Uchida M, Sawada H, Nakamura T, Maekawa N, Uchino H: Ingestion and killing of bacteria by neutrophils from patients with leukemia—a new method for evaluating phagocyte function at short incubation periods. Acta Haematol Jpn 45:56, 1982

17. Hirschberg N: Phagocytic activity in leukemia. Am J Med Sci 197:706, 1939

18. Solberg CO, Schreiner A, Hellum KB, Hamre E: Neutrophil granulocyte function in the early diagnosis of acute myelomonocytic and myeloblastic leukaemia. Acta Med Scand 197:147, 1975

19. Wantzin GL, Wantzin J: The NBT test: Erratic behaviour in acute leukaemia. Blut 31:133, 1975

20. Ohta H: Metabolic responsiveness of human leukemic leukocytes to phagocytic stimulation. Acta Haematol 33:28, 1965

21. Pickering LK, Anderson DC, Choi S, Feigin RD: Leukocyte function in children with malignancies. Cancer 35:1365, 1975

22. Humbert JR, Hutter JJ, Jr, Thoren CH, DeArmey PA: Decreased neutrophil bactericidal activity in acute leukemia of childhood. Cancer 37:2194, 1976

23. Heilmann C, Pedersen FK: Granulocyte function in children

with acute lymphoblastic leukaemia during remission. Scand J Haematol 25:30, 1980

24. Koch C: Heterogeneity of phagocytic malfunction in myeloid leukaemia. Localization of the primary defect to decreased intracellular killing by immature myeloid cells. Acta Pathol Microbiol Scand (sec C) 83:383, 1975

25. Thompson EN, Williams R: Bactericidal capacity of peripheral blood leucocytes in relation to bacterial infections in acute lymphoblastic leukaemia in childhood. J Clin Pathol 27:906, 1974

26. Goldman JM, Th'ng KH: Phagocytic function of leukocytes from patients with acute myeloid and chronic granulocytic leukaemia. Br J Haematol 25:299, 1973

27. Wilkinson PM, Sumner C, Delamore IW, Geary CG, Milner GR: Granulocyte function in myeloblastic leukaemia. Br J Cancer 32:574, 1975

28. Rosner F, Valmont I, Kozinn PJ, Caroline L: Leukocyte function in patients with leukemia. Cancer 25:835, 1970

29. Baehner RL, Neiburger RG, Johnson DE, Murrmann SM: Transient bactericidal defect of peripheral blood phagocytes from children with acute lymphoblastic leukemia receiving craniospinal irradiation. N Engl J Med 289:1209, 1973

30. Uden AM, Palmblad J, Venizelos N: Neutrophil function during immunotherapy for acute myelogenous leukemia in remission. Nouv Rev Fr Hematol 24:231, 1982

31. Silver RT, Beal GA, Schneiderman MA, McCullough NB: The role of the mature neutrophil in bacterial infections in acute leukemia. Blood 12:814, 1957

32. Gallin JI: Human neutrophil heterogeneity exists, but is it meaningful? Blood 63:977, 1984

33. Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE: Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. J. Immunol 136:860, 1986

34. Keston AS, Brandt R: The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal Biochem 11:1, 1965

35. Bass DA, Parce JW, DeChatelet LR, Szejda P, Seeds MC, Thomas M: Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J Immunol 130:1910, 1983

36. Seeds MC, Parce JW, Szejda P, Bass DA: Independent stimulation of membrane potential changes and the oxidative metabolic burst in polymorphonuclear leukocytes. Blood 65:233, 1985

Selective Inhibition of the Growth of Human Erythroid Bursts by Monoclonal Antibodies Against Transferrin or the Transferrin Receptor

By Kevin M. Shannon, James W. Larrick, Samuel A. Fulcher, Kathy B. Burck, John Pacely, Jack C. Davis, and David B. Ring

The relative requirements of colonies derived from erythroid (BFU-E) and myeloid (CFU-c) progenitors for transferrin were examined using monoclonal antibodies directed against the transferrin molecule (TF-6) or its cell surface receptor (TFR-A12, TFR1-2B). Growth of erythroid bursts was profoundly reduced at concentrations of all three antibodies that had no effect on CFU-c-derived colonies. When TFR1-2B was layered over cultures established one to seven days previously, further burst development was inhibited, and degeneration of early erythroid colonies was observed. Addition of erythropoietin augmented transferrin receptor expression on cells harvested after 1 to 2 weeks in culture and analyzed by flow cytometry. Recombinant human erythropoietin gave results comparable to those obtained in experiments using human urinary erythropoietin. Analysis of erythroblasts plucked directly from culture plates confirmed the presence of transferrin receptors on BFU-E-derived colonies. Thymidine incorporation was maximal early in the second week of culture and coincided with high transferrin receptor expression. These data demonstrate that transferrin must be available into the second week of culture to support the growth and differentiation of BFU-E-derived erythroid bursts, that the generation of erythroid colonies from BFU-E is more dependent on transferrin than myeloid colony formation from CFU-c, and that erythropoietin modulates the expression of transferrin receptors on growing bursts.
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TRANSFERRIN'S function as the major blood protein engaged in iron transport has been known for many years.[1] The observation that it is an essential constituent of serum-free culture media[2] has stimulated interest in transferrin's role as a cellular growth factor. Actively proliferating cells express a high density of surface receptors for transferrin,[3-5] and monoclonal antibodies (MoAb) that specifically bind to these receptors inhibit the growth of transformed cell lines and of myeloid progenitor colonies (CFU-c).[6,7]

In culture systems developed during the past decade, erythroid progenitors form distinctive colonies and can be induced to differentiate in vitro under the influence of erythropoietin (EPO).[8,9] Erythroid bursts, which are derived from early progenitors termed burst-forming units (BFU-E), can readily be enumerated from human blood and bone marrow mononuclear cells.[9,10] Availability of these clonogenic assays for erythroid progenitors has facilitated investigation of factors that regulate erythropoiesis. Although transferrin receptors have been identified on erythroid precursors present in human bone marrow[11] and on erythroblasts in mature BFU-E,[12] work reported to date has not addressed the importance of transferrin receptor expression during the growth and differentiation of erythroid bursts.

Our interest in the effects of transferrin on erythroid progenitors was stimulated by demonstration of an autoantibody against the transferrin receptor in a woman with severe microcytic anemia.[13] Serum from that patient blocked the uptake of radiolabeled iron and fluorescein isothiocyanate-labeled transferrin by K562 cells and inhibited surface binding of MoAb against the transferrin receptor to target cells.[13] The observation that this patient maintained adequate WBC and platelet counts suggested that normal erythropoiesis requires relatively higher levels of bioavailable transferrin. In the present study we show (1) that MoAb against transferrin or its cell surface receptor abrogate the growth of erythroid progenitor colonies derived from BFU-E at much lower concentrations than are required to inhibit CFU-c development, (2) that the requirement of developing bursts for transferrin extends into the second week of culture, and (3) that EPO increases proliferation and expression of transferrin receptors on cultured umbilical cord mononuclear cells.

MATERIALS AND METHODS

Production and characterization of MoAb. MoAb TF-6, which is specific for human transferrin, was produced by standard fusion techniques. Murine plasmacytoma cell line NS1 was fused to splenocytes from mice immunized and boosted with iron-saturated human transferrin. Hybridomas were screened by transferrin-specific enzyme-linked immunosorbent assay (ELISA). Cloned cell line TF-6 produced an IgG monoclonal antibody that immunoprecipitates human transferrin. The antibody also recognized transferrin bound to cell surface.

MoAb TFR1-2B[13] and TFR-A12 (originally designated 454-A12),[14], which recognize the transferrin receptor, have been described previously. Both antibodies bind to cell surface transferrin receptors, immunoprecipitate the 94,000-molecular weight (mol wt) transferrin receptor, and recognize different sites on the receptor distant from the actual transferrin binding site. TFR1-2B is of the IgG2a isotype and TFR-A12 of the IgG1 isotype. Ascites containing TF-6, TFR-A12, and TFR1-2B was purified by Staph-Protein A affinity chromatography and by diethylaminoethyl (DEAE) ion-exchange chromatography.

MoAb MY7[15] was obtained from Coulter Immunology (Hialeah, Fla). A MoAb that recognizes DR antigens was obtained from Becton Dickenson (Mountain View, Calif).

Culture of myeloid and erythroid progenitors. Approximately 10 mL of whole blood was collected from the cut end of the

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umbilical cords of healthy neonates into 10 mL of heparinized culture medium consisting of 20% fetal calf serum and 1% bovine serum albumen in RPMI 1640 medium (GIBCO, Grand Island, NY). Samples were kept at room temperature for up to 16 hours prior to isolation of mononuclear cells by Ficoll-Hypaque density gradient centrifugation. Mononuclear cells were aspirated from the interface, and the remaining erythrocytes were lysed in ammonium chloride solution. After washing, the cell population was resuspended in Iscove's modified Dulbecco's medium (IMDM) for plating in culture medium consisting of 0.8% methylcellulose and supplemented with 35% fetal calf serum (Flow Laboratories, McLean Va; lot 29101036), 1.2% bovine serum albumen, L-glutamine, and final concentrations of 10^{-5} Mol/L 2-mercaptoethanol and 0.01% iron-free human transferrin (Sigma Chemical Co, St Louis). Commercial colony-stimulating factor (GCT medium, GIBCO; lot 18K6242) was added to all cultures. Human urinary erythropoietin was obtained from the British Columbia Cancer Centre, Vancouver, BC. In some experiments, recombinant human erythropoietin with a specific activity of > 70,400 U/mg of protein (Amgen Corp, Thousand Oaks, Calif) was used instead of EPO purified from human urine. Duplicate 30 × 15-mm plates (Falcon 3001, Oxnard, Calif) containing 1 mL of methycellulose culture medium and a final concentration of 5×10^4 cells per plate were cultured. After 14 days at 37 °C in a 5% CO_2 incubator, progenitor colonies were enumerated by indirect light microscopy according to the criteria of Gregory and Eaves for erythroid bursts[9] and Pike and Robinson for CFU-c–derived colonies.[16]

A number of precautions were taken to ensure consistency of the culture conditions. Single lots of fetal calf serum and GCT medium were used in all experiments. Stock methylcellulose culture medium was prepared in 100-mL lots, tested for its ability to support the growth of BFU-E and CFU-c, and stored at −70 °C in test tubes containing 2.5 mL each before use. For individual experiments, all cord blood mononuclear cell cultures were carried out on the same lot of methylcellulose medium. Antibody was diluted and added directly to culture tubes along with GCT medium, cell suspension, and EPO. The tubes were mixed thoroughly from one to three hours before duplicate 1-mL plates were prepared. For experiments in which the antibody TFR1-2B was added from one to seven days after the cultures had been established, 100 μL of antibody diluted in IMDM was carefully layered over each 1-mL plate.

To assess the effects of early antibody exposure on colony development, a series of experiments was carried out in which primary cultures established as previously described were incubated at 37 °C from one to five days. These cells were then harvested and replated in secondary cultures as follows: primary dishes were washed vigorously with 15 mL of IMDM and centrifuged at 2,000 rpm for 15 minutes. The pellets were next washed three times in a large excess of IMDM to dilute out residual antibody and methylcellulose. Following the final wash, the cell pellet was resuspended in 0.3 mL of IMDM and added to 2.5 mL methylcellulose tubes from the same lot, mixed, and replated. Secondary plates were scored for the presence of BFU-E– and CFU-c–derived colonies 14 days after the original cultures had been established.

All studies using human umbilical cord blood were approved by the Committee for the Protection of Human Subjects at the Naval Hospital, Oakland, Calif, and by the Naval Health Sciences Education and Training Command.

Preparation of mononuclear cells for immunofluorescent analysis. Cultured cells were removed from methylcellulose after seven to 14 days by serially washing 35-mm plates with a total of 12 mL of medium. Cells were centrifuged at 1,500 rpm for ten minutes, and the pellet was resuspended in IMDM and washed twice to remove any residual methylcellulose. Mononuclear cells obtained in this manner were used in studies of surface antibody binding and thymidine incorporation as will be described. Purified populations of erythroblasts were obtained by plucking individual bursts under indirect microscopy using a 10-μL micropipette. Wright-Giemsa staining confirmed that more than 95% of cells obtained in this manner were erythroblasts.

Cell sorter analysis. An EPICS V sorter (Coulter Electronics, Hialeah, Fla) equipped with a 76-μm nozzle and a 5-W argon laser was used for cell surface marker analysis. The 488 nmol/L line at 1-W light-regulated power was used for fluorescein excitation. Cells were stained with monoclonal antibodies by the method of Loken and Stall.[17] Fluorescein isothiocyanate conjugate goat F $(ab')_2$ anti-γ and anti-μ reagents were purchased from Tago (Burlingame, Calif). To eliminate dead cells from analysis, 10 μL of propidium iodide (Sigma Chemical Co, 5 μg/mL in distilled water) was added to 1 mL of cells in phosphate-buffered saline (PBS) with 0.1% gelatin and 0.2% NaN_3, and fluorescence was measured on propidium iodide (PI)-negative cells. The percentage of cells labeled by individual antibodies was determined by a computer program that uses a least-squares algorithm for immunofluorescent histogram analysis (Coulter Electronics, Hialeah, Fla).

Proliferation assay. Mononuclear cells were removed from methylcellulose and washed as previously described. Cells were immediately transferred to flat-bottomed microtiter plates (1×10^5 cells per well) and incubated for six hours at 37 °C in the presence of tritiated thymidine (0.5 μCi/well) before harvesting. Wells were aspirated using a PHD cell harvester (Cambridge Technology), and Scintiverse scintillation fluid (Fisher Scientific, Fairlawn, NJ) was added to vials, which were then counted on a Minibeta 1212 (LKB, Gaithersburg, Md) liquid scintillation counter. A total of six wells was cultured in all proliferation studies; the results of individual experiments were obtained by eliminating the highest and lowest counts and calculating a mean for the four remaining wells.

Statistical analysis. The effect of various concentrations of TF-6, TFR1-2B, and TFR-A12 on colony growth was assessed by comparing the growth in plates containing antibody with plates prepared in parallel from the same cord blood specimen to which no antibody was added. Growth in plates without antibody was defined as representing 100% of control numbers for that experiment and was compared with the growth in plates to which antibody had been added. The mean percent inhibition and SD were calculated by pooling the data derived from individual experiments. All statitical comparisons were made using Student's paired *t* test.

RESULTS

Growth of progenitors in the absence of antibody. Cord blood mononuclear cells cultured in the presence of 2U EPO/mL without antibody yielded a mean of 146 CFU-c (range 51 to 254) and 122 BFU-E (range, 23 to 220) derived colonies per 10^5 cells plated. The plating efficiency for erythroid progenitors grown from cord blood mononuclear cells was similar to the results of others[10] and to what we have reported previously.[18] In the absence of added EPO, no BFU-E colonies were observed while myeloid colony growth was unchanged.

Selective inhibition of BFU-E growth by MoAb. Addition of TFR1-2B at the time cultures were established profoundly reduced the growth of BFU-E–derived colonies as shown in panel A of Fig 1. Erythroid colony numbers were reduced to less than 20% of the control by 0.1 μg/mL of TFR1-2B; no bursts were observed at concentrations >0.5 μg/mL. In contrast, a significant decrease in the number of

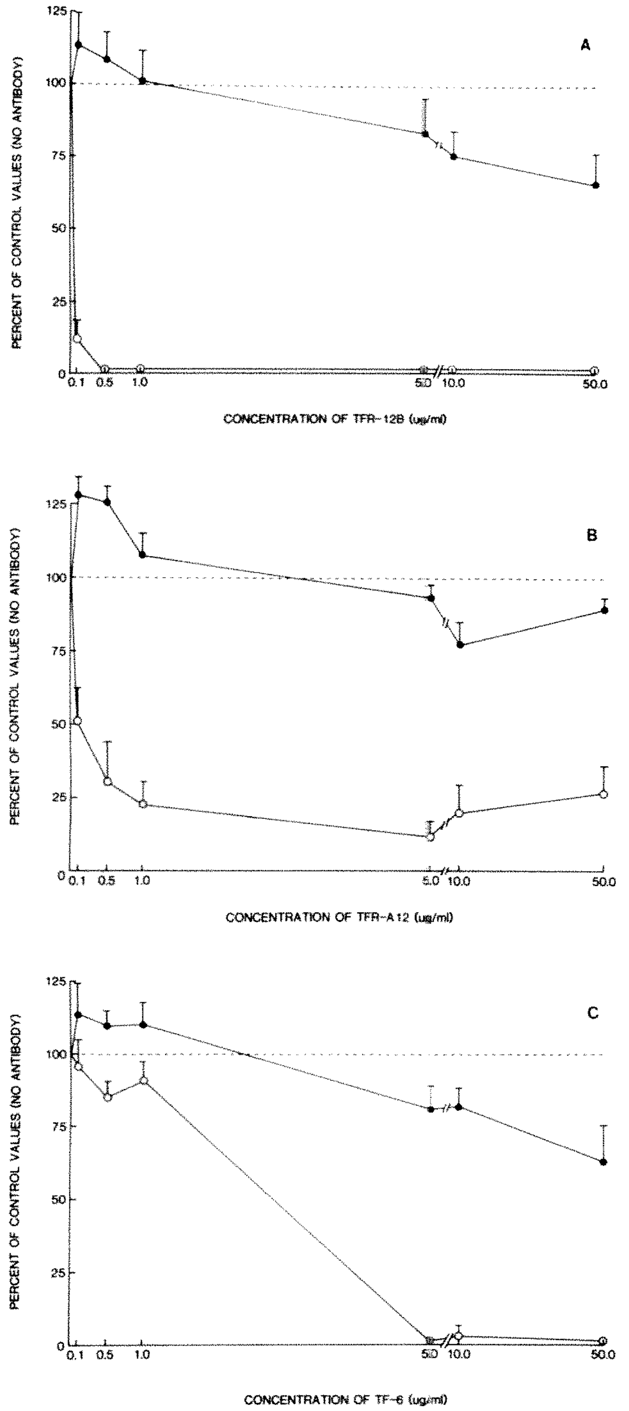

Fig 1. Effect of MoAb on progenitor colony growth. At final concentrations between (A) 0.1 and 50.0 μg/mL, TFR1-2B, and (B) TFR-A12 significantly reduced BFU-E–derived colony growth ($P < .001$ compared with control cultures for all doses tested). Development of bursts proceeded normally in the presence of 0.5 and 1.0 <μg/mL of TF-6; (C) however, 5.0 μg/mL completely abrogated erythroid colony growth. All three antibodies profoundly decreased the number of bursts at concentrations that had no effect on CFU-c–derived colonies. Some reduction of CFU-c numbers occurred at 10.0 and 50.0 μg/mL ($.01 < P < .05$ for all three antibodies). Each point represents pooled data from five to 13 experiments. ●=CFU-C; O=BFU-E.

CFU-c–derived colonies did not occur until a TFR1-2B concentration of 10.0 μg/mL was reached. Even at 50.0 μg/mL of antibody, growth of CFU-colonies remained greater than 50% of control values.

A somewhat different pattern of inhibition was observed in cultures to which TFR-A12 was added (Fig 1, panel B). As was the case with TFR1-2B, erythroid bursts were far more sensitive to the inhibitory effects of antitransferrin receptor antibody than were CFU-c–derived colonies. Reduction in CFU-c colony numbers occurred at TFR-A12 concentrations of 10.0 and 50.0 μg/mL ($.01 < P < .05$), but overall growth was greater than 50% of control values, even at the highest dose of antibody tested. In contrast, low doses of TFR-A12 (0.1 to 0.5 μg/mL) decreased BFU-E–derived colonies by 50% to 80%. Most of the erythroid colonies present in cultures carried out in the presence of these low doses of antibody were large and contained abundant hemoglobin. Morphologically, they were similar to the well-developed burst shown in the first panel of Fig 2. At concentrations of TFR-A12 above 1.0 μg/mL, the number of bursts did not decrease further; however, the erythroid colonies present were poorly developed and contained very little visible hemoglobin. A representative burst grown in the presence of 5.0 μg/mL of TFR-A12 is shown in the second and third panels of Fig 2.

Results of experiments using TF-6, the MoAb against the transferrin molecule, are summarized in panel C of Fig 1. The effects on colony growth of antibody against transferrin were similar to those of antibodies against its cell surface receptor. Development of bursts was inhibited at significantly lower concentrations of TF-6 than that of CFU-c. Growth of erythroid progenitors was normal at 0.1 and 1.0 mg/mL of TF-6 but was completely abrogated by 5.0μg/mL. A significant decrease in the number of CFU-c was not observed until an antibody concentration of 10.0 μg/mL was reached, and growth remained greater than 60% of control plates at 50.0 μg/mL.

Kinetics of BFU-E inhibition by TFR1-2B. Addition of antibodies against transferrin or its cell surface receptor at the time cultures were established markedly reduced the proliferation and differentiation of erythroid progenitors, as was shown. We next investigated the effect of deferring the addition of antibody until one to seven days after the cells were initially plated. For these experiments, we selected a concentration of TFR1-2B (3.0 μg/mL) that was known to completely inhibit the development of BFU-E–derived colonies if added on day 0. We observed a >90% reduction in the number of bursts when TFR1-2B was added as late as day 7 (Fig 3). This finding is especially notable because immature bursts containing few cells and small amounts of hemoglobin become visible between day 5 and day 7. Most of these colonies had degenerated by day 14 in plates to which TFR1-2B had been added. The observation that transferrin must be available into the second week of culture for optimal growth and development of BFU-E–derived progenitor colonies led us to conduct experiments in which mononuclear cells were harvested from methylcellulose to analyze transferrin receptor expression and proliferation.

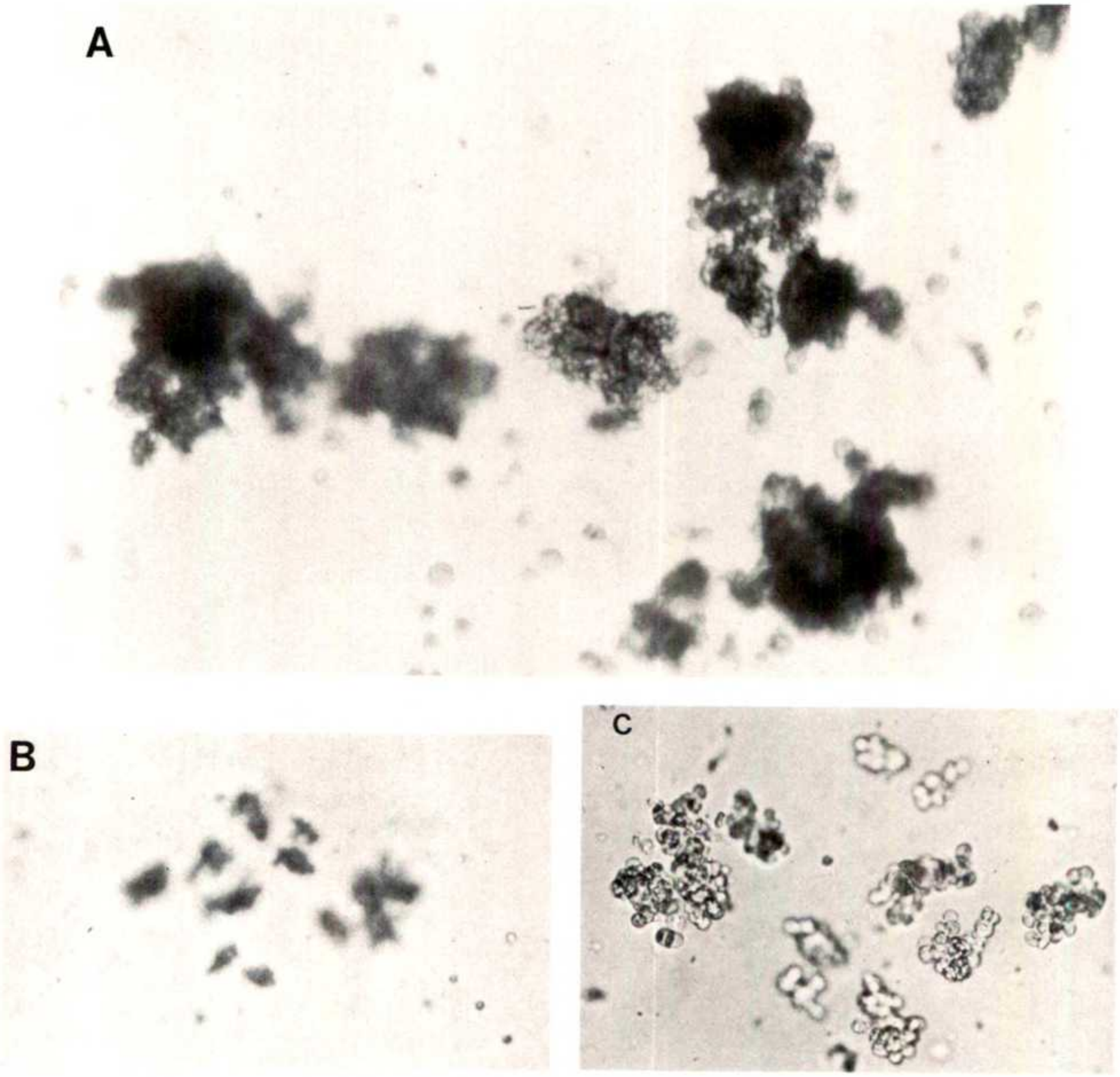

Fig 2. Alteration of burst morphology by TFR-A12. (A) normal erythroid burst containing large subcolonies appears dark because of abundant hemoglobin production (50×). (B) Representative burst from the same cord blood specimen grown in the presence of 5 μg/mL of TFR-A12 (50×). Individual bursts were poorly developed and contained small subcolonies (C). Higher power view of burst from (B) (original magnification × 2(100×); current magnification). Subcolonies contain few cells; little hemoglobin was visible in individual colonies.

Replating experiments and preincubation with MoAb. In the experiments just described, antibodies were consistently present in all cultures throughout the second week, a period of rapid proliferation and hemoglobinization in developing bursts. To more directly assess the sensitivity of BFU-E to antitransferrin receptor antibodies early in culture, mononuclear cells were harvested after 1, 3, or 5 days, washed, and replated in secondary cultures. For these studies, final concentrations of TFR-A12 and TFR1-2B of 1.0 μg/mL were used in both primary and secondary cultures. As shown in Table 1, inhibition of BFU-E colony development was dependent upon the duration of antibody exposure. Cultures that contained TFR1-2B or TFR-A12 for one day yielded normal numbers of BFU-E at 2 weeks. In contrast, when replating was delayed as long as five days, a marked reduction in BFU-E growth was observed (Table 1). The few colonies that were derived from secondary cultures established on day 5 without antibody from plates initially cultured with antibody were poorly developed and resembled those shown in the second and third panels of Fig 2. Growth of CFU-c was completely unaffected by these relatively low concentrations of antibody and was similar in cultures replated throughout the first five days.

Because the methylcellulose medium used to culture progenitor colonies (35% fetal calf serum) contained large amounts of transferrin, it was possible that the pronounced

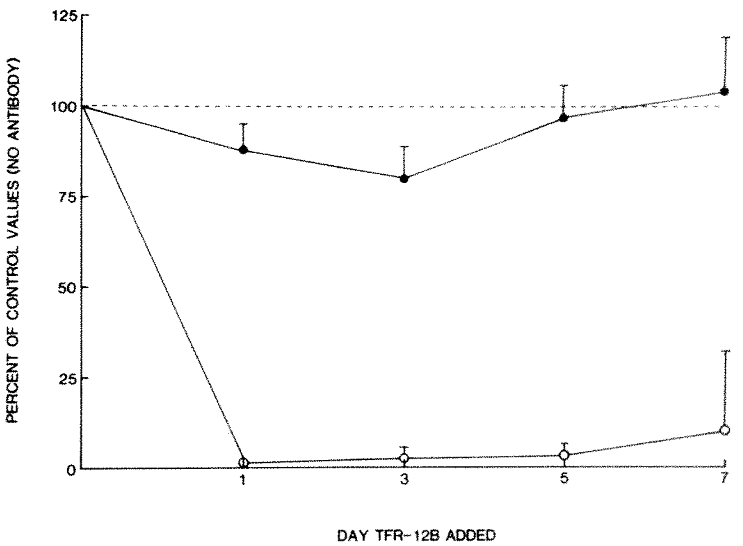

Fig 3. The addition of a final concentration of TFR1-2B of 3 μg/mL between one and seven days after the establishment of cultures profoundly inhibited the growth of BFU-E-derived colonies ($P < .001$ for all days). The data were pooled from six experiments. ●=CFU-C; ○=BFU-E.

difference in the growth of erythroid and myeloid colonies might be due to a rapid uptake of transferrin by CFU-c rather than to a true difference in the intrinsic sensitivity of the target cells to MoAb. To investigate this possibility, cord blood mononuclear cells were incubated overnight in IMDM containing either no antibody or 10 μg/mL of TF-6, TFR1-2B, or TFR-A12. The cells were then washed thoroughly and cultured in methylcellulose. Preincubation of mononuclear cells with antibody resulted in no inhibition of either erythroid or myeloid colony growth at 2 weeks unless additional antibody was added at the time of plating in methylcellulose (data not shown).

Observations using the antibody TF-6, which does not bind to cell membranes, suggested that our results could not be explained by nonspecific, complement-mediated cytotoxicity. To further evaluate this question, methylcellulose medium was heat inactivated for two hours at 56 °C immediately prior to the addition of cells, antibody, and EPO. The inhibitory effects of TFR1-2B, TFR-A12, and TF-6 on the growth of BFU-E–derived colonies were identical in control and heat-inactivated media (data not shown).

MoAb antibody binding and proliferative response of cultured mononuclear cells. Surface labeling of transferrin receptors and of the myeloid differentiation antigen MY7 was carried out on cells harvested from methylcellulose. Addition of EPO at the time cultures were established markedly increased the expression of transferrin receptors on mononuclear cells analyzed after seven to 14 days (Table 2). In contrast, the higher proportion of MY7-positive cells in cultures grown without EPO reflects a predominance of myeloid progenitor colonies. Histograms from an experiment in which cells from a single cord blood specimen were labeled with TFR1-2B and MY7 after nine and 14 days in culture are presented in Fig 4. Both the percentage of cells with detectable transferrin receptors and the density of labeling with antireceptor antibodies were greater in cultures harvested on day 9. Because the major difference between cultures grown with and without EPO was the presence of BFU-E–derived colonies in the former, we inferred that erythroid progenitors were the population with high transferrin receptor expression. This was confirmed by analysis of erythroblasts plucked from mature bursts after 14 days in culture (data not shown).

Table 1. Growth of Erythroid Bursts Following Replating

Antibody Added (+) to		Number of BFU-E Colonies per 10^5 Cells in Cultures Replated on		
Primary Cultures	Replated Cultures	Day 1 (n = 6)	Day 3 (n = 2)	Day 5 (n = 7)
–	–	133 ± 28	72 ± 2	123 ± 24
+	–	122 ± 22	36 ± 12*	33 ± 14‡
–	+	26 ± 8†	11 ± 11*	13 ± 6†
+	+	16 ± 7‡	0 ± 0‡	3 ± 1‡

Symbols indicate values significantly different from reference cultures replated on the same day containing no antibody in either primary or replated cultures. Data from these reference cultures are plotted on the top line. * = $P < .05$; † = $P < .01$; ‡ = $P < 0.005$. Values for day 1 and day 5 were analyzed by the paired *t* test; values for day 3 were from one-way analysis of variance (ANOVA) because of the small number of data points.

Table 2. Surface Binding of Antibody by Cord Blood Mononuclear Cells

Experiment	Days in Culture	Percentage Positive With Antibody			
		TFR1-2B	TFR-A12	MY7	Anti-DR
1	7 +EPO*	32.2	27.0	24.3	9.7
	–EPO	1.7	1.3	44.0	19.3
2	7 +EPO	33.3	ND	14.0	ND
	–EPO	3.7	ND	24.0	ND
3	9 +EPO	84.4	87.3	4.4	ND
	–EPO	14.4	ND	29.5	ND
3	14 +EPO	26.7	28.6	0.6	ND
	–EPO	0.7	1.4	6.6	ND
4	14 +EPO	17.5	31.8	7.9	ND
	–EPO	0.3	ND	15.7	ND
5	14 +EPO	62.0	ND	ND	7.0
	–EPO	16.0	ND	ND	39.0

Abbreviation: ND, phenotype not determined.

* +EPO and –EPO refer to cells removed from plates cultured in the presence (+) or absence (–) of erythropoietin.

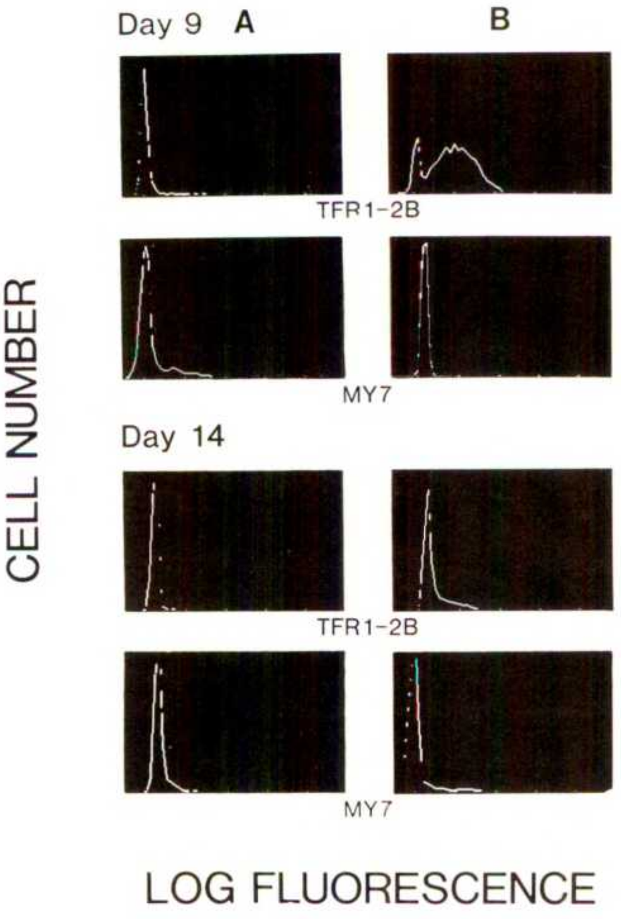

Fig 4. Flow cytometry of cells grown with (A) EPO– and without (B) EPO– erythropoietin. The heavy line represents binding by fluorescein-labeled TFR1-2B or MY7; the light line shows a fluorescein-labeled negative control antibody. Transferrin receptor expression was markedly increased on cells harvested from EPO$^+$ plates; in contrast, a higher percentage of EPO– cells were labeled by MY7. Enumeration of progenitor colonies on day 14 revealed 74 CFU-c– and 110 BFU-E–derived colonies per 10^5 cells cultured on EPO$^+$ plates and 76 CFU-c and no BFU-E colonies on EPO– plates. Since cells from the same cord blood were analyzed after nine and 14 days in culture, the data also suggest downregulation of transferrin receptors on mature bursts. The percentage of positive cells in each study is given in Table 2 as experiment 3.

To determine whether the increase in transferrin receptor expression in the presence of EPO was accompanied by cellular proliferation, thymidine uptake by cultured cells was examined. As depicted in Fig 5, maximum thymidine incorporation was observed in plates containing EPO on days 9 and 11; this correlated with a high level of transferrin receptor expression on cells harvested on day 9 (Fig 4).

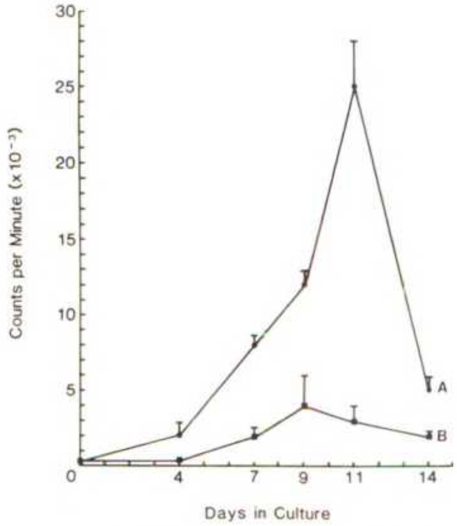

Fig 5. Proliferation of cells grown with (A) EPO$^+$ and without (B) EPO– erythropoietin. Serial analysis of thymidine incorporation of cells cultured from a single cord blood specimen pulsed after four to 14 days in culture. The high level of thymidine incorporation early in the second week of culture coincides with a rapid increase in the cellularity and hemoglobinization of bursts. Flow cytometry of cells harvested from the same experiment (Fig 4) showed that transferrin receptor expression on days 9 and 14 paralleled changes in thymidine uptake.

Because human urinary EPO is only partially purified, it is possible that the enhancement of cell proliferation and of surface transferrin receptor expression was due to a contaminating factor in the preparation and not EPO. To address this question directly, cells from individual patients were cultured in the presence of both urinary and recombinant EPO. Results of a representative experiment are given in Table 3. The similar effects of both EPO preparations suggest that EPO is the molecule responsible for the augmentaion of proliferation and transferrin receptor expression observed in culture.

DISCUSSION

Although investigation of a variety of cell lines has established a relationship between transferrin receptor density and proliferation[3,7,19-21] little experimental information is available regarding transferrin receptor expression during the growth and differentiation of normal cells. Erythroid maturation is of particular interest because of the large iron requirement for hemoglobin synthesis. We have shown that monoclonal antibodies that recognize transferrin or its surface receptor profoundly decrease the growth of erythroid progenitors at concentrations that have no effect on myeloid colony development.

Burst development is regulated by the combined effects of burst-promoting activity (BPA) and EPO.[22,23] Early proliferation and burst formation is dependent on BPA and proceeds in the absence of EPO.[22] Erythropoietin is required for later differentiation and for hemoglobinization of maturing bursts. We found that when mononuclear cells initially cultured in the presence of low concentrations of antitransferrin receptor MoAb TFR-A12 and TFR1-2B were removed from methylcellulose and replated at three or five days, the number of BFU-E–derived colonies was markedly reduced, suggesting that transferrin is necessary early in burst development. Other experiments showed that when antibody addition was deferred for as long as one week, proliferation was arrested, and degeneration of the immature colonies occurred. The additional demonstration that cells

Table 3. Comparative Effects of Urinary and Recombinant EPO on Cultured Cells

Erythropoietin Added (U/mL)	Days in Culture	Percentage Positive With TFR1-2B	TFR-A12	MY-7	Thymidine Uptake (cpm ± SEM)
No EPO	10	4.1	4.0	38.3	1,609 ± 334
	14	0.0	0.0	19.4	2,501 ± 195
Urinary EPO (2 U/mL)	10	89.4	87.3	1.1	9,046 ± 577
	14	61.8	48.0	1.0	12,254 ± 1,071
Recombinant EPO (2 U/mL)	10	73.2	79.8	4.3	6,569 ± 1,130
	14	60.0	42.0	6.4	14,244 ± 713

express a high density of surface transferrin receptors well into second week in culture also suggests that transferrin is required during both EPO-dependent and EPO-independent stages of burst maturation. In contrast, cells that were either preincubated in IMDM containing 10 μg/mL of antireceptor antibody or that were removed from cultures containing antibody after a single day and replated yielded normal numbers of BFU-E colonies when washed and plated without antibody. These results suggest that BFU-E have low transferrin receptor expression in vivo that increases in response to culture conditions that promote differentiation of progenitors into mature bursts.

An interesting finding was that although both antireceptor antibodies reduced erythroid colony development at low concentrations, their dose-response effects were somewhat different. As little as 0.5 μg/mL of TFR1-2B completely abrogated burst growth. In contrast, the addition of between 0.1 and 1.0 μg/mL of TFR-A12 decreased erythroid progenitor colony growth in a dose-related fashion. Increasing the concentration of TFR-A12 above 1.0 μg/mL did not further reduce the number of colonies; however, the bursts that were present contained few cells and visibly less hemoglobin. The mechanism by which TFR1-2B and TFR-A12 inhibit cellular proliferation has not been established. These antibodies recognize separate sites on the transferrin receptor that are distinct from the binding site for transferrin.[13,14] Uptake of transferrin-bound iron by K562 cells is unaffected by either TFR-A12 or TFR1-2B.[13] We have recently shown that fluorescein-labeled TFR1-2B remains bound on the surface of K562 target cells at 37 °C; subsequent binding of transferrin to the receptor triggers endocytosis of the antibody-transferrin-receptor complex.[24] Transferrin and TFR1-2B are co-internalized and accumulate in the same perinuclear cluster of vesicles.[19,24] We have no evidence as yet that TFR1-2B alters recycling of the transferrin receptor to the cell surface or inhibits the release of iron from vesicles, although either mechanism might explain its effects on the growth of BFU-E–derived colonies. The antitransferrin antibody TF-6, which does not bind to cell membranes, produced selective inhibition of erythroid colony growth similar to that observed with TFR-12B and TFR-A12. This finding is important because it suggests that the antireceptor antibodies act specifically by depriving growing cells of transferrin. Our observation that a discrete threshold concentration of TF-6 completely abrogates burst formation is consistent with a previous report that cell lines will undergo maximal proliferation only when a critical amount of transferrin is available to them.[21]

Whether proliferation is triggered by the accumulation of sufficient intracellular iron or is due to some other aspect of the interaction of transferrin with its receptor is unclear. It is attractive to hypothesize that erythroid cells, which need large amounts of iron to synthesize hemoglobin, will only undergo clonal expansion when a critical amount of intracellular iron is present. Recent evidence suggests that intracellular iron levels are crucial in regulating the expression of transferrin receptors on growing cell lines.[20] Taetle and associates[25] investigated the requirement of myeloid leukemia cells for transferrin in serum-free culture medium. Exogenous soluble iron reversed the inhibitory effects of a MoAb against the transferrin receptor on cell growth, and increased intracellular ferritin concentrations were noted in cells that were able to grow in the presence of antibody.[25] We and others[12] have shown that erythroblasts plucked from bursts have a higher density of surface transferrin receptors than cells from CFU-c–derived colonies; yet we found that erythroid colony formation is inhibited by lower concentrations of monoclonal antibodies that bind transferrin or its receptor. The observation that the proliferation and differentiation of erythroid progenitors, which possess a higher density of transferrin receptors than myeloid cells, is inhibited by much lower concentrations of MoAb suggests that development of erythroid bursts from BFU-E requires that many more transferrin molecules be bound to the receptor than are necessary for CFU-c growth. Our results are consistent with the hypothesis that erythroid progenitors must accumulate relatively high intracellular levels of iron to carry out the differentiation program to mature bursts and that this accounts for their sensitivity to antibodies that recognize transferrin or its surface receptor.

We have shown that EPO increases proliferation and transferrin receptor expression by cultured mononuclear cells and that this coincides with morphologic maturation of erythroid bursts. Our findings in studies of normal human progenitors are consistent with a preliminary report that observed that EPO increased both the synthesis of transferrin receptors and their expression on Friend virus–transformed cells.[26] It is likely that the augmentation of iron uptake induced by EPO that has previously been observed in mouse erythroleukemia (Friend) cells[27] is modulated through increased expression of surface transferrin receptors.

Erythroid progenitors both express a high density of surface transferrin receptors and are extremely sensitive to inhibition by monoclonal antibodies against transferrin or its receptor. Our findings suggest that transferrin plays a major regulatory role in the process by which BFU-E differentiate into bursts. Further study will be required to characterize the relationship between intracellular iron levels and burst growth and to precisely define the effects of EPO on the expression and function of transferrin receptors present on normal erythroid cells.

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REFERENCES

1. Aisen P, Brown EB: Structure and function of transferrin. Prog Hematol. 9:25, 1975
2. Barnes D, Sato G: Serum-free cell culture: A unifying approach. Cell 22:649, 1980
3. Larrick JW, Cresswell P: Modulation of cell surface iron transferrin receptors by cellular density and state of activation. J Supramo Struc 11:579, 1979
4. Mendelsohn J, Trowbridge I, Castagnola J: Inhibition of human lymphocyte proliferation by monoclonal antibody to transferrin receptor. Blood 62:821, 1983
5. Neckers LM, Cossman J: Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. Proc Natl Acad Sci USA 80:3494, 1983
6. Trowbridge IS, Lopez F: Monoclonal antibody to transferrin receptor blocks transferrin binding and inhibits human tumor cell growth in vitro. Proc Natl Acad Sci USA 79:1175, 1982
7. Taetle R, Honeysett JM, Trowbridge I: Effects of antitransferrin receptor antibodies on growth of normal and malignant myeloid cells. Int J Cancer 32:343, 1983
8. Clarke BJ, Housman D: Characterization of an erythroid precursor of high proliferative capacity in normal human peripheral blood. Proc Natl Acad Sci USA 74:1105, 1977
9. Gregory CJ, Eaves AC: Human marrow cells capable of erythropoietic differentiation in vitro: Definition of three colony responses. Blood 49:855, 1977
10. Weinberg RS, Goldberg JD, Schofield JM, Lenes AL, Stczynski R, Alter BP: Switch from fetal to adult hemoglobin is associated with a change in progenitor cell population. J Clin Invest 71:785, 1983
11. Horton MA: Expression of transferrin receptors during erythroid maturation. Exp Cell Res 144:361, 1983
12. Sieff C, Bicknell D, Robinson J, Lam G, Greaves MF: Changes in surface antigen expression during erythropoietic differentiation. Blood 60:703, 1982
13. Larrick JW, Hyman ES: Acquired iron-deficiency anemia caused by an antibody against the transferrin receptor. N Engl J Med 311:214, 1984
14. Bjorn MJ, Ring D, Frankel A: Evaluation of monoclonal antibodies for the development of breast cancer immunotoxins. Cancer Res 45:1214, 1985
15. Griffin JD, Ritz J, Beveridge RP, Lipton JM, Daley JF, Schlossman SF: Expression of MY7 antigen on myeloid progenitor cells. Int J Cell Cloning 1:33, 1983
16. Pike BL, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76:77, 1970
17. Loken MR, Stall AM: Flow cytometry as an analytical and preparative tool in immunology. J Immunol Methods 50:85, 1982
18. Shannon K, Davis J, Kitzmiller J, Fulcher S, Koenig H: Erythropoiesis in the infants of diabetic mothers. Pediatr Res 20:161, 1986
19. Enns CA, Larrick JW, Suomalainen H, Schroder J, Sussman HH: Co-migration and internalization of transferrin and its receptor on K562 cells. J Cell Biol 97:579, 1983
20. Louache F, Testa U, Pelicci P, Thomopoulos P, Titeux M, Rochant H: Regulation of transferrin receptors in human hematopoietic cell lines. J Biol Chem 259:11576, 1984
21. Raso V, Basala M: A highly cytotoxic human transferrin–ricin A chain conjugate used to select receptor-modified cells. J Biol Chem 259:1143, 1984
22. Tsang RW, Aye MT: Evidence for proliferation of erythroid progenitor cells in the absence of added erythropoietin. Exp Hematol 7:383, 1979
23. Adamson JW, Torok-Storb B, Lin N: Analysis of erythropoiesis by erythroid colony formation in culture. Blood Cells 4:89, 1978
24. Larrick JW, Enns C, Raubitschek A, Weintraub H: Receptor-mediated endocytosis of human transferrin and its cell surface receptor. J Cell Physiol 124:283, 1985
25. Taetle R, Rhyner K, Castagnola J, To D, Mendelsohn J: Role of transferrin, Fe, and transferrin receptors in myeloid leukemia cell growth. J Clin Invest 75:1061, 1985
26. Sawyer ST, Krantz SB: Effect of erythropoietin on transferrin receptor sites, synthesis, and cyling in maturing Friend virus–infected cells. Blood 64:135, 1984 (abst)
27. Koury MJ, Bondurant MC, Duncan DT, Krantz SB, Hankins WD: Specific differentiation events induced by erythropoietin in cells infected in vitro with the anemia strain of Friend virus. Proc Natl Acad Sci USA 79:635, 1982

Biology of Human Megakaryocyte Factor V

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To learn more about human megakaryocyte coagulation cofactor V (FV), we studied the expression of this protein in normal bone marrow megakaryocytes and in megakaryocytes cloned from their colony-forming unit in FV-depleted plasma clot cultures. Mouse monoclonal antibodies directed against either the light chain or an activation peptide of human FV and a rabbit polyclonal, monospecific FV antiserum were used as probes for these experiments in conjunction with a variety of immunochemical detection techniques. All morphologically recognizable megakaryocytes were shown to contain FV. The origin of this protein appeared to be both from FV bound to the cell as well as from endogenous FV in the majority of cells examined. The existence of a population of small bone marrow mononuclear cells that simultaneously expressed platelet glycoproteins and FV was also noted. Such cells represented ~70% of all small cells positive for platelet glycoproteins. In contrast, only about 40% of megakaryocyte colonies cloned in FV-deficient medium contained cells with immunochemically detectable FV. FV expression was most clearly demostrated in large cells in the colonies, whereas smaller, presumably less mature cells labeled weakly or not at all. Synthesis of FV by human megakaryocytes was documented using elutriation-enriched cells incubated in ^{35}S-methionine-containing medium. Megakaryocyte lysates and medium conditioned by these cells were subjected to immunoaffinity column purification. Column eluates analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography revealed radioactive bands comigrating with the heavy and light chains of thrombin-activated FV. These studies suggest that human megakaryocytes both bind and synthesize FV. Expression of these traits appears to be related to cell maturation, with binding ability appearing earlier than the ability to synthesize this protein. Finally, although the ability to bind FV appears to be universal among megakaryocytes, our culture data suggest that synthesis may be a restricted, or constitutively expressed property of these cells.

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COAGULATION cofactor V (FV) is a high-molecular weight glycoprotein that performs several critical functions in regulating hemostasis. In its activated form (FVa), FVa interacts with factor Xa and Ca^{++} to form the prothrombinase complex.[1] This complex, when assembled on the platelet[2] or other phospholipid-containing surface[3] catalyzes the formation of thrombin from prothrombin at rates up to 278,000 times faster than factor Xa alone.[4] FVa also serves as one cofactor in the activation of protein C by thrombin in the fluid phase[5] and on the surface of the endothelial cells.[6] Protein C is known to inactivate FVIIIa and FVa.[7] Thus, FV exerts an important regulatory role in whole blood coagulation.

The origin of the FV, which is immunocytochemically detectable on human megakaryocytes[8,9] has not yet been determined. Some could be bound by these cells since FVa is known to bind to human platelets.[10,11] Alternatively, FV could be synthesized by megakaryocytes. This hypothesis is tenable in view of the fact that guinea pig megakaryocytes have been shown to synthesize FV[12] as well as a number of other coagulation-related proteins.[13,14] In addition, Chesney et al have observed that unless human platelets are stimulated FV appears to be localized to their α-granules and is not found on the cell surface.[15] The possibility that a combination of bound plus synthesized material accounts for discernible human megakaryocyte FV also needs consideration. Since Tracy and co-workers have recently suggested that human plasma FV and platelet FV are functionally distinct entities, with the latter as well as the former being obligate requirements for normal hemostasis,[16] the answer to this question has assumed new significance. If it can be shown that human megakaryocytes synthesize the FV found in megakaryocyte and platelet α-granules and the functional heterology of plasma and platelet FV is confirmed, them megakaryocytes play an even more important role in the process of coagulation than has heretofore been appreciated. We therefore studied human megakaryocytes to learn more about the origin and biology of FV associated with these cells.

MATERIALS AND METHODS

Sources of human megakaryocytes: bone marrow smears. After obtaining informed consent, marrow specimens were obtained from patients undergoing bone marrow aspiration as part of their diagnostic evaluation. Marrows were smeared, air-dried, fixed immediately in methanol:acetone (1:9), and stored frozen at −20 °C until needed.[17]

Isolated bone marrow megakaryocytes. Human bone marrow obrained from the femurs of patients undergoing total hip replacement was collected in the megakaryocyte collection medium described by Berkow et al,[18] itself a modification of the CATCH medium (calcium- and magnesium-free Hanks' balanced salt solution containing 10^{-3}, mol/L adenosine, 2×10^{-3} mol/L theophylline, 3.8% sodium citrate, and 3.5% bovine serum albumin) described by Levine and Fedorko.[19] The medium of Berkow was further modified by the addition of lidocaine (5×10^{-5} mol/L) and prostaglandin ($PGE_{1\alpha}$) (2×10^{-7} mol/L) (Sigma Chemical Co, St. Louis) to inhibit neutrophil and platelet release reactions, respectively. DNAse I was deleted from the medium.

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Marrow tissue was finely minced in the megakaryocyte collection medium and then filtered through a 100-μm pore sized nylon filter (Nytex, Tetko, Inc, Elmsford, NY) to remove bone particles and cell clumps. The cell suspension was pelleted by centrifugation at 200 g for eight minutes. The collection medium was then aspirated, and the red cells in the pellet were lysed with Lysing Reagent (Ortho Diagnostic Systems, Raritan, NJ). On some occasions, red blood cells were removed by resuspending packed marrow cells to a hematocrit value of 50% in megakaryocyte collection medium and then further diluting these cells in 3% dextran (Clinical Grade, Sigma Chemical Co) at a final ratio of one part cell suspension to two parts dextran. Cells were then allowed to sediment for 20 minutes.[20]

Leukocytes depleted of red cells were filtered through another 100-μm nylon filter and then subjected to counterflow centrifugal elutriation using a Beckman J2-21 M centrifuge equipped with a JE 6 Rotor and standard elutriation chamber. Flow through the rotor was controlled with a Masterflex pump (Cole Parmer, Chicago). Approximately 5×10^8 cells were loaded into the separation chamber by a gravity feed system, rather than by injection, so that the cells could be loaded at a low concentration with a minimum of mechanical manipulation. Rotor speed during loading was 1,000 rpm with a pump flow rate of 7 mL/min. Immediately after loading all the cells, the rotor speed was decreased to 800 rpm, and the counterflow pump rate was increased to 15 mL/min. These conditions were maintained until cells ceased issuing from the separation chamber. At that time, the counterflow pump was turned off and the rotor stopped. The megakaryocyte-rich cell preparation was then removed from the separation chamber by gentle aspiration. Total time from loading to removal of cells was usually ~ 1.5 hours.

Elutriated cells were further purified by density gradient centrifugation with Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) (1.050 g/mL). After centrifugation at 750 g for 25 minutes, cells above and collecting at the Percoll gradient interface were gently removed by aspiration and washed once in the megakaryocyte collection medium. Cell counts were performed with an electronic particle counter (model Fn, Coulter Electronics, Hialeah, Fla). From ~ 5×10^8 total nucleated cells, we routinely obtained from 1×10^5 to 2×10^5 megakaryocytes in cell suspension of ~50% purity. Megakaryocyte-rich cell suspensions were then deposited onto glass slides for immunochemical studies using International Equipment Corporation (IEC, Needham Heights, Mass) cytobuckets, or labeled with ^{35}S-methionine as will be described.

Cultured megakaryocytes. The culture system used has been previously described.[21,22] Bone marrow mononuclear cells used for cell cloning were obtained from marrow specimens removed from femurs during the course of total hip replacement. All human serum used in the cultures were depleted of FV as will be described.

In some cases, marrow mononuclear cells were cloned in NU-SERUM (Collaborative Research, Lexington, Mass), a medium supplement containing 15.1 mg protein/mL. The only human constituent of this formulation is transferrin (50 μg/mL). At the concentration used, NU-SERUM was determined to contain approximately 0.65 μg/mL of material cross-reactive with FV antigen or ~ 6% of the normal human plasma concentration. This value was obtained using an enzyme-linked immunosorbent assay (ELISA) that we have developed for measurement of human FV antigen.[23]

Beef plasma was used as a source of fibrinogen for clotting of cultures and comprised 9% of the total plated volume. The ELISA assay[23] was able to detect 250 ng/mL of cross-reacting FV antigen in undiluted beef plasma whereas the known human plasma concentration is ~ 10 μg/mL. Therefore, the amount of immunodetectable FV antigen contributed to cultures by the beef plasma was 23 ng/mL or less than 0.25% of the normal human plasma concentration.

Factor V–depleted human serum. Human serum added to the megakaryocyte culture medium, whether derived from normal AB donors or aplastic anemia patients, was rendered deficient of FV coagulant activity and antigen by passage through an immobilized immunosorbent of rabbit polyclonal, monospecific antibody to human FV prepared as previously described.[23] This procedure resulted in <0.001 μg/mL of detectable FV remaining in the serum by the coagulant assay and <0.3 μg/mL of FV antigen using the ELISA assay. Since the concentration of human serum in the cultures was 27% of the total plated volume, the final FV antigen concentration in serum so treated was <.090 μg/mL or less than 1% of the normal human plasma concentration.[23]

Rabbit antiserum against human platelet glycoprotein (PGP). The preparation of this antiserum has been previously described.[17,24] It has been shown to be highly specific for reactivity with PGP and does not react with monocytes.[17,21]

Rabbit antiserum against human factor V. Preparation and purification of this reagent has been previously described.[23] The final product neutralizes FV and displays a single peak on crossed immunoelectrophoresis of normal plasma.

Mouse monoclonal antibodies (MoAb) against human factor V. BALB-c mice, 3 to 4 months old, were immunized with purified human FV[23] (75 μg) intraperitoneally with complete Freund's adjuvant and boostered with 50 μg in saline 14 days later. Three days later, their spleens were aseptically removed, and the cells (10^8/mL) were fused with P3-×63/Ag 8.653 mouse myeloma cells (10^7/mL) in 38% polyethylene glycol (molecular weight (Mol wt) = 1,450) for one minute at 37 °C. After centrifugation for five minutes at 200 g, 3 mL of serum-free Dulbecco's modified Eagle's medium (GIBCO, Chagrin Falls, Ohio) was added. The cells were then washed and resuspended in hypoxanthine, aminopterin, and thymidine (HAT) medium, 15% fetal calf serum, gentamicin (0.5%) and L-glutamine (2 mmol/L). They were then cloned by limiting dilution, distributed in 96 wells of Linbro tissue culture plates (Flow Laboratories, McLean, Va), and cultured for 2 weeks. The culture fluids were tested for antibody by adding 100 μL to polystyrene cuvettes precoated with FV. After washing, bound antibody was detected using the ELISA assay.[23]

Cultures producing anti–factor V antibodies were recloned twice by limiting dilution. Strongly positive clones were then selected for propagation in the ascites of pristane-primed mice. Purification of these antibodies was achieved by precipitation in 40%-saturated ammonium sulfate solution followed by affinity chromatography on diethyl aminoethyl (DEAE) Affi-gel blue (Bio-Rad, Richmond, Calif).

The MoAb were typed by reacting the supernatants of the ascites fluid or hybridoma cell culture medium with solid-phase FV coated on the surface of cuvettes. Following this, specific rabbit antibodies to different mouse immunoglobulin subclasses were added to the cuvettes along with alkaline phosphatase–conjugated goat antirabbit IgG. A solution of *P*-nitrophenyl phosphate was added as a chromogenic substrate, and absorbance at 405 nm was measured. Fresh tissue culture medium and normal mouse IgG were used as negative controls.

The ability of the MoAb to neutralize FV was determined by the ability of cloned antibodies to inhibit clot formation in a FV-specific clotting assay[25] as previously described.[12]

Epitope-binding specificity of the derived antibodies was determined by immunoaffinity gel chromatography.[12] The MoAb whose specificity was to be defined was coupled to cyanogen bromide–activated Sepharose 4B as previously described.[12] Purified FV,[23] radiolabeled by the iodogen method[26] and cleaved by human α-thrombin[23] was then applied to the immunoaffinity column after washing and equilibration with a 0.02 mol/L Tris buffer, pH 7.2, containing 0.15 mol/L NaCl. Any nonspecifically bound proteins were washed from the column with 0.37 mol/L NaCl, pH 7.2, containing 0.02 mol/L Tris buffer followed by a rinse with 0.15 mol/L NaCl in 0.02 mol/L Tris buffer containing 5% glycerine. Specifically bound protein (~40% of total counts applied to the column) was then eluted with 0.02 mol/L Tris buffer containing 20 mmol/L EDTA, 0.15 mol/L NaCl, 25% glycerine, and 50% ethylene glycol. The mol wt of the reduced, bound material was determined by sodium dodecyl sulfate (SDS) slab gel electrophoresis.[27] A 3% to 15% gradient polyacrylamide gel was used. Purified FV and mol wt standards (Bio-Rad) consisting of myosin (200,000), β-galactosidase (116,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), and purified fibrinogen (330,000) were also applied to the gels as references.The dried gel was subjected to autoradiography with Kodak X-Omat AR x-ray film (Eastman Kodak Co, Rochester, NY) for 2 weeks at -80 °C.

Immunofluorescence. The procedure used has been previously described.[17,22] For detection of binding of rabbit immunoglobulins in single-label experiments, a fluorescein isothiocyanate–conjugated goat antirabbit IgG was used. For detection of binding of mouse immunoglobulin, a fluorescein isothiocyanate–conjugated sheep antimouse IgG antiserum was used. All conjugated antisera were obtained from Sigma Chemical Co.

Bone marrow megakaryocytes were identified by their bright fluorescence, large size, and multiple nuclear lobes. Small fluorescein-positive marrow cells were defined as intensely fluorescent cells whose diameters were ≤ 18 μm as measured with a JENA optical fylar (Ausjena, Jena, GDR) and whose nuclei were visible by either fluorescence or phase microscopy.

A dual-label antibody immunofluorescence technique was sometimes used so that the expression of two different phenotypic markers by a single cell could be assayed simultaneously. For positive megakaryocyte identification, the specimens to be studied were first labeled with rabbit anti-PGP antiserum diluted 1:200 in phosphate-buffered saline (PBS).[17] They were then washed and incubated in the purified mouse monoclonal anti-FV antibody (B38) for three hours at 37 °C. After washing, fluorescein isothiocyanate–conjugated sheep antimouse IgG and tetramethylrhodamine isothiocyanate–conjugated goat antirabbit IgG were added simultaneously at a titer of 1:40. The fluorescence staining procedure was then completed as previously described,[17,21,22] save that specimens were not counterstained with Evan's blue dye.

Immunoperoxidase. Specimens were fixed as previously described and rinsed in PBS containing 1% bovine serum albumin, (PBS/BSA), which was used for all subsequent rinses unless specifically noted. Normal rabbit or normal mouse serum (40 μL in 10 mL PBS) was added to the specimen for 30 minutes and rinsed away. Mouse monoclonal anti-FV antibody (0.1 μg IgG/mL in PBS) or control mouse ascites (Cappel Laboratories, Malvern, Pa) at the same concentration was then layered over the specimen for 30 minutes. Following another rinse, endogenous peroxidase activity was quenched by the addition of 0.6% H_2O_2 (Fisher Scientific Co, King of Prussia, Pa) in CH_3OH. Biotinylated antimouse IgG antibody (Vector Laboratories, Burlingame, Calif) was then layered over the specimen. After rinsing avidin-biotin-horseradish peroxidase complex (Vector Laboratories) was added, followed by the addition of 0.06% diamino-benzidine tetrahydrochloride (Kodak), the peroxidase substrate. Gill's hematoxylin (Fisher Scientific, King of Prussia, Pa) was used as a nuclear counterstain and saturated Li_2CO_3 (Sigma Chemical Co) solution as a bleaching solution.

Cells cultured in plasma clots were stained in situ. They were scored as positive if their membranes were delineated by a dark brown granular ring and/or if their cytoplasm contained a distinct granular brownish-black material. Cells were scored as negative if they had a bluish-grey or faint brown cytoplasm that was not clearly distinguishable from that of other cells in the cultures or the background. Eosinophil colonies, which were easily identified by their highly granular, nonrefractile cytoplasm and growth in tight clusters, were scored negatively because of an inability to quench their endogenous peroxidase activity.

To quantify the amount of peroxidase substrate accumulated in individual bone marrow megakaryocytes, a Zeiss SF microscope photometer (Carl Zeiss, Oberhausen, West Germany) interfaced to a Zonax controller system (Carl Zeiss) was used to allow for computer-assisted data analysis and storage. Photometric measurements were made in the density mode. Density units were arbitrary. Glass slides upon which the elutriated megakaryocytes were deposited were placed on the microscope stage after immunoperoxidase staining. The microscope was focused at a total magnification of 100×. One hundred percent density (7.6 on the arbitrary scale) was then defined as the measurement recorded when no light was allowed to reach the photometer whereas 0% density was set to correspond to that measurement recorded over the darkest background area of the glass slide. Individual megakaryocytes were then visualized through a target reticle at 100× total magnification. The visually densest part of the cytoplasm, corresponding to an area ~8μm in diameter, was then photometrically measured.

Immunoaffinity gel chromatography of radiolabeled megakaryocyte proteins. Approximately 2×10^5 megakaryocytes, enriched by centrifugal elutriation, were suspended in 1 mL of serum-free, methionine-free Dulbecco's minimal essential medium (MEM) (GIBCO Laboratories) containing 500 μCi of ^{35}S-methionine (Amersham Corp, Arlington Heights, Ill). After 15 hours, incubation in a 37 °C shaking water bath, the cells were washed three times in PBS and then resuspended in 0.5 mL of PBS containing BSA (0.1%), Triton X-100 (Sigma, St Louis) (0.1%) and the following protease inhibitors: phenylmethylsulfonylfluoride (2mmol/L), soybean trypsin inhibitor (2.4 μmol/L), benzamidine chloride (1 mmol/L), aprotinin (100 kIU/mL), leupeptin (50 μmol/L), and Eglin (CIBA-Geigy, Basel, Switzerland) (1 mg/mL). Cells were lysed in this buffer by sonication and repeated freeze-thawing. The resulting lysates were clarified by centrifugation in an Eppendorf microfuge (Brinkman Instruments, Westbury, NY). Clarified lysates were then applied to a mouse monoclonal antihuman FV (B38) affinity column and eluted as previously described save that the elution buffer contained calcium and no EDTA. Eluates were analyzed on a 1.5-mm continuous gradient (5% to 15%) SDS-polyacrylamide gels and then subjected to autoradiography. As a control, an equal number of leukocytes derived from the Percoll purification step were radiolabeled, lysed, and analyzed in exactly the same way. Gels were exposed to x-ray film for 20 days at -80 °C. A Lightening PLUS intensifying screen (DuPont, Wilmington, Del) was used.

Photomicroscopy. An Olympus BH-2 reflected light fluorescence microscope (Olympus, New Hyde Park, NY) equipped with a PM-10AD photomicrographic system was used. The PM-10AD unit automatically determined and set optimal film exposure once ASA, and film reciprocity information was entered into the unit.

RESULTS

Mouse MoAb recognizing human factor V. After screening supernatants from multiple hybridomas, one clone was identified that contained antibody with a high degree of FV-binding ability as determined by ELISA. This clone, designated B38, was also able to completely neutralize FV coagulant activity at a titer of greater than 1/12,800. Another hybridoma, designated B10, also displayed high FV-binding ability (titer, >1/12,800) but had no neutralizing activity, even at a titer of 1:10. Both clones were subsequently expanded in the ascites of pristane-primed mice.

Characterization of the MoAb obtained from the ascitic fluid with regard to immunoglobulin type and FV binding site specificity was carried out as described in Materials and Methods. Both MoAb were determined to have a IgG1 heavy chain and a κ light chain.

Thrombin-cleaved FV was found to yield four polypeptide fragments when electophoresed in the polyacrylamide gels. These fragments corresponded in mol wt to the previously described heavy chain (D fragment, mol wt ~105,000), light chain (F_1, F_2 fragments, mol wt ~71 to 74,000), and activation C peptide (mol wt ~150,000).[28] In addition, a fragment of mol wt ~54,000 was also noted.[28] MoAb B38 was found to recognize only the light chain of FVa (mol wt ~74,000) as evidenced by the fact that in the presence of EDTA only the light chain bound to the column (Fig 1). This result is consistent with its ability to neutralize FV (Va) activity. Using similar methodology, MoAb B10 was found to recognize only the ~150,000–mol wt activation peptide (Fig 2) of FV, a finding consistent with its inability to inactivate FV.

Factor V expression by human bone marrow megakaryocytes. We initially sought to determine whether FV was associated with megakaryocytes by immunocytochemical means. For these studies, methanol:acetate-fixed, elutriation-enriched human bone marrow megakaryocytes were used. These preparations were probed with (1) a well-characterized rabbit antihuman FV antiserum, (2) the same antiserum absorbed with purified human FV until all FV neutralizing activity was lost, and (3) normal rabbit serum. All working antiserum dilutions were equivalent. Binding of antibodies was determined using an immunoperoxidase detection technique. Examination of the megakaryocytes after labeling with the rabbit antihuman FV antiserum revealed that virtually all these cells were densely labeled in comparison with the small contaminating cells in the background (Fig 3A). Specificity of this reaction was confirmed by demonstrating that megakaryocytes incubated with FV-absorbed rabbit anti-FV antiserum (Fig 3B) or with normal rabbit serum (Fig 3C) displayed little or no staining in comparison with both contaminating background cells and cells labeled with the rabbit antihuman FV antiserum.

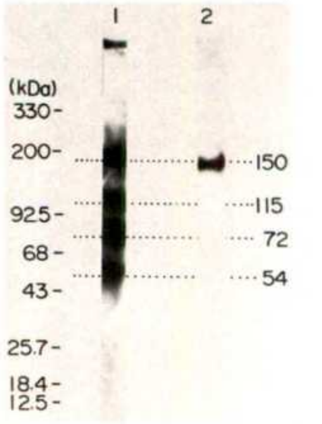

Fig 2. Epitope-binding specificity of mouse MoAb B10. The procedures used are described in Materials and Methods and briefly recapitulated in the legend of Fig 1. Autoradiograph of the dried gel is shown. Numbers on the left indicate migration positions of known mol wt standards applied to the gel. Numbers on the right show the apparent mol wt of indicated bands. Lane 1 demonstrates the migration pattern (in kilodaltons [kDa]) of proteolytic fragments of thrombin-activated FV. Lane 2 demonstrates the location of the single band, mol wt ~150,000, eluted from the B10 column in the presence of 20 mmol/L EDTA. This band corresponds in mol wt to the C activation peptide of FV.[27]

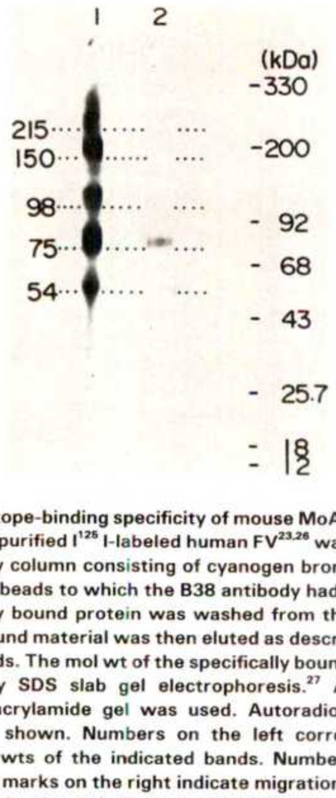

Fig 1. Epitope-binding specificity of mouse MoAb B38. Thrombin-activated, purified I^{125} I-labeled human FV[23,26] was applied to an immunoaffinity column consisting of cyanogen bromide–activated Sepharose 4B beads to which the B38 antibody had been coupled. Nonspecifically bound protein was washed from the column, and specifically bound material was then eluted as described in Materials and Methods. The mol wt of the specifically bound material was determined by SDS slab gel electrophoresis.[27] A 3% to 15% gradient polyacrylamide gel was used. Autoradiographs of the dried gel are shown. Numbers on the left correspond to the apparent mol wts of the indicated bands. Numbers with corresponding dash marks on the right indicate migration of known mol wt standards in the gel. Lane 1 demonstrates the migration pattern of proteolytic fragments of thrombin-activated FV added directly to the gel. Lane 2 demonstrates that only one protein was specifically bound to the column. The mol wt of this single band, ~75,000, corresponded to the light chain (F_1, F_2 fragment) of FV seen in lane 1. The elution buffer was devoid of calcium and contained EDTA (20 mmol/L) to dissociate the light and heavy chains of factor Va.

We then attempted to determine whether immature, nonmorphologically recognizable megakaryocytes also expressed FV. Human bone marrow smears were used for this purpose since elutriation experiments were designed to result in the preferential elimination of small cells from specimens. After determining that nonspecific fluorescence of marrow megakaryocytes was minimal when double labeled with normal rabbit serum and control mouse ascites as described in Materials and Methods (Figs 4A, B, C), we then performed dual-labeling experiments with a rabbit antiserum recognizing human platelet glycoproteins as a specific megakaryocyte lineage marker and the B38 mouse MoAb.

Marrow slides were first examined under green light for the identification and enumeration of PGP-bearing small cells. Once identified, these cells were then observed under

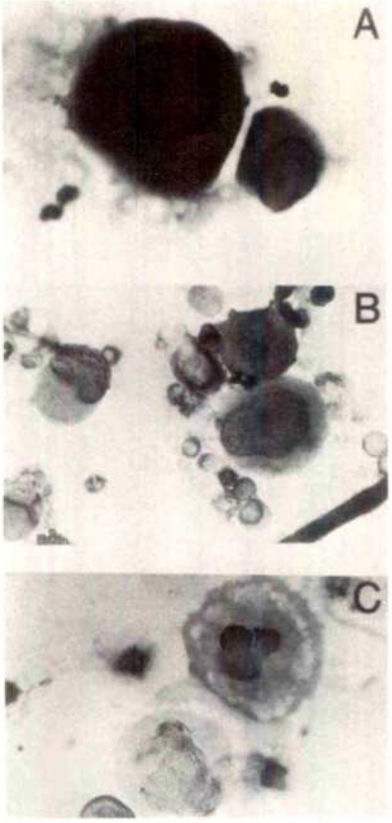

Fig 3. Immunochemical detection of FV on elutriation-enriched human bone marrow megakaryocytes. Megakaryocytes were deposited onto glass slides by cytocentrifugation, fixed with methanol:acetone (1:9), and then reacted with a polyclonal-monospecific rabbit antihuman FV antiserum (panel A). Binding of probe antibody was demonstrated with an immunoperoxidase detection technique. The same procedure was then carried out with an antiserum absorbed with purified FV until all FV neutralizing activity had been removed. Results obtained are depicted in panel B. For comparison purposes, panel C demonstrates labeling achieved with normal rabbit serum used at an equivalent serum dilution. The largest megakaryocyte in (A) measured 62 μm in diameter; in (B), 40 μm; and in (C), 51 μm.

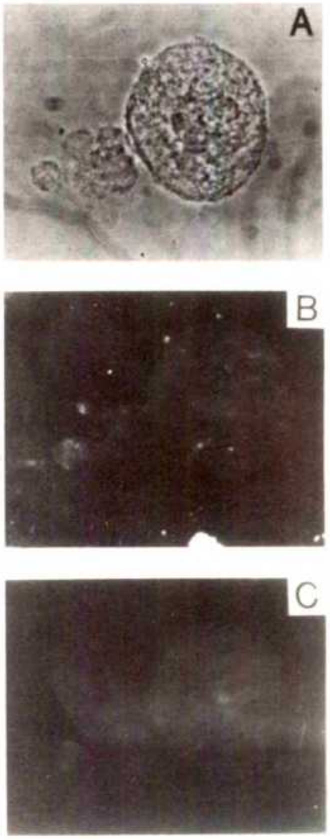

Fig 4. Fluorescence of elutriation-enriched human marrow megakaryocytes reacted with normal rabbit serum and control mouse ascites plus fluorescein-conjugated sheep antimouse IgG and tetramethylrhodamine-conjugated goat antirabbit IgG. A high-power, phase-contrast view of cells is shown in (A). A large megakaryocyte is shown on the right, with contaminating leukocytes shown clumped to its left. Fluorescence of cells viewed under blue light is shown in (B) and under green light in (C). Similar results were obtained when fluorescein-conjugated goat antirabbit IgG and tetramethylrhodamine-labeled goat antimouse IgG were used. The megakaryocyte featured measured ~53 μm in diameter.

blue light to determine whether they also expressed FV. Ninety-three PGP-positive cells were identified in a total of three different experiments. Sixty-five (70%) of these cells simultaneously expressed FV (Figs 5A, B, C) whereas 28 (30%) were found to be FV-negative. Morphologic, and in particular, nuclear differences between FV positive and negative groups were searched for to determine whether the apparent level of small-cell maturation[29] might have been responsible for the observed differences, but no obvious distinctions were noted.

The labeled marrow specimens were also examined to determine whether other cell types expressed FV, but except for platelets, megakaryocytes, and the small cells just described, no other marrow elements labeled with the anti-FV antibodies.

Labeling of human bone marrow megakaryocytes with monoclonal anti-human FV MoAb of different epitope specificities. In an attempt to determine whether the aforementioned FV expression by megakaryocytes was due to the binding of FV from plasma and/or the result of endogenous synthesis, we labeled preparations of elutriation-enriched megakaryocytes with either a mouse MoAb recognizing the activation peptide (mol wt ~150,000) of FV (B10) or one recognizing the light chain (mol wt ~74,000) of FVa (B38) in conjunction with the immunoperoxidase technique. For control purposes, similar preparations were labeled with a control mouse ascites containing an equivalent amount of normal mouse IgG. After examining several hundred cells, we observed that cells reacted with control ascites displayed little labeling, cells reacted with B38 had dense labeling, and cells reacted with B10 had a label density between the two. This qualitative assessment was objectified by measuring the stain accumulation over individual megakaryocytes as a function of density using a computer-interfaced microspectrophotometer (Zonax, Zeiss). A total of 99 megakaryocytes were examined in each group. The mean ± SD of the

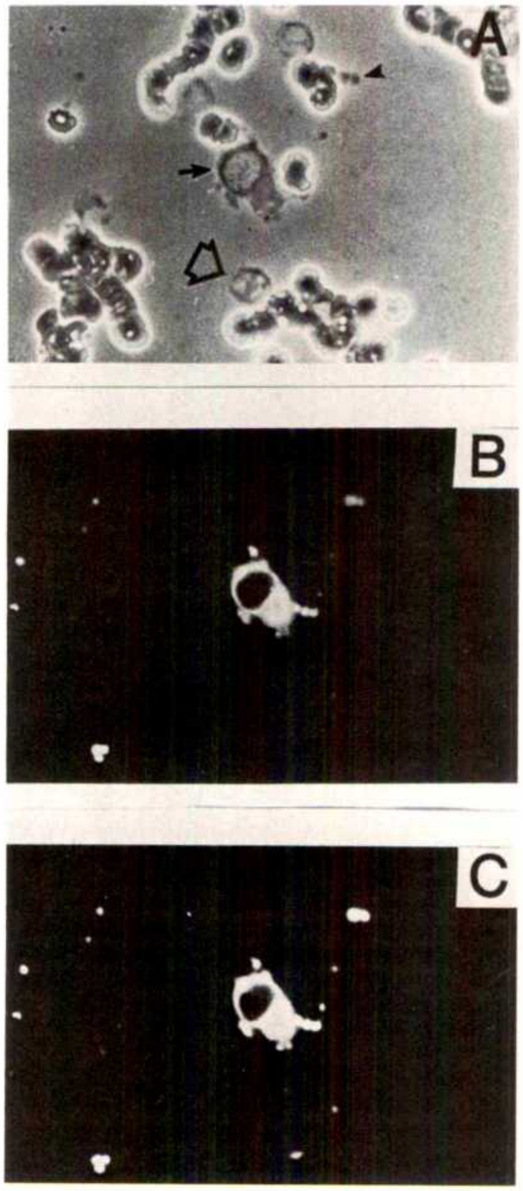

Fig 5. Simultaneous expression of PGP and FV by small bone marrow mononuclear cells. A methanol:acetone-fixed bone marrow smear was dual labelled with rabbit antihuman PGP antiserum and mouse antihuman FV MoAb as detailed in Materials and Methods. A phase-contrast photomicrograph of an immature megakaryocyte (small arrow) is shown in A. Note also in A platelets (arrow head) and a granulocyte (large open arrow). When viewed under green light, small megakaryocyte and platelets fluoresced brightly, indicating expression of PGP (B). The small megakaryocyte and platelets also fluoresced brightly under blue light, indicating expression of FV (C). In neither instance did the granulocyte exhibit significant fluorescence. The immature megakaryocyte featured measured ~15 μm at its greatest diameter.

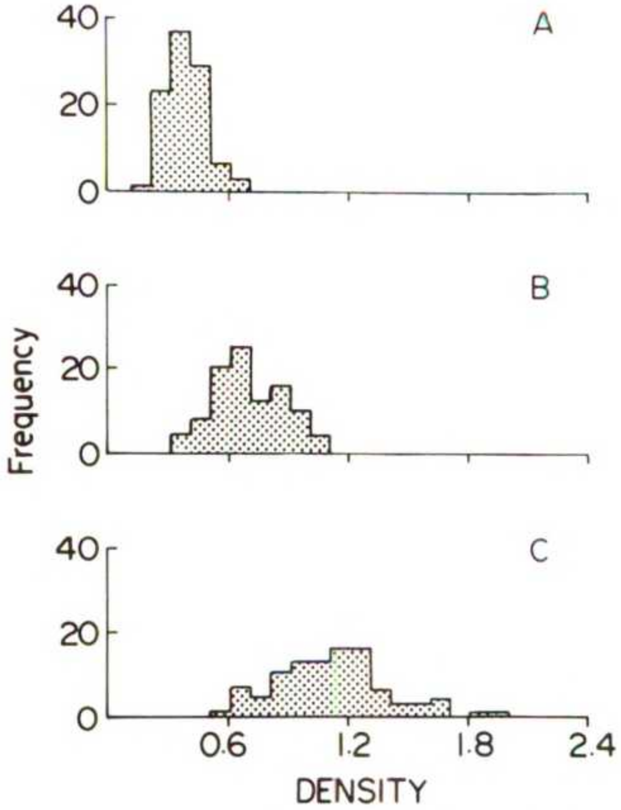

Fig 6. Computer-generated frequency histograms of density measurements gathered on elutriation-enriched bone marrow megakaryocytes. Cells were probed with control mouse ascites (A), B10; activation peptide (mol wt ~150,000)–specific, MoAb (B); or B38, light-chain (mol wt ~74,000)–specific, MoAb (C). Binding of the probe antibodies was determined using an immunoperoxidase detection technique as detailed in Materials and Methods. Protein concentrations were equivalent. Ninety-nine individual megakaryocytes were analyzed in each group. Measurements were computer assigned to arbitrary density classes incremented in arbitrary density units of 0.1. The number of measurements per class is displayed.

measured density for each experimental group was generated by the computer and displayed as frequency histograms (Fig 6).

The Zonax-generated data analysis revealed that megakaryocytes labeled with control ascites had a mean density of .377 (± .09) with 97% of the cells having a density of 0.6 or less (Fig 6, histogram A). The mean density of megakaryocytes labeled with the B10 antibody was .694 (± 0.18). Examination of the density class distributions of cells labeled with B10 (histogram B) revealed that 32.2% of the cells examined had a density of 0.6 or less. These cells were therefore classified as being negatively stained, whereas the remaining 67.8% of the cells were classified as positive for FV. A density class distribution analysis of cells labeled with B38 disclosed that 99% had a density greater than 0.6 with the mean density of this group being 1.1 (± 0.27).

FV expression in cultured human megakaryocytes. Human bone marrow light-density (≤1.077 g/mL) mononuclear cells were cloned in plasma clot cultures immunodepleted of almost all detectable FV (≤6% of normal human plasma concentration). After fixation, the cultures were probed with the rabbit antihuman FV antibody to identify any FV-positive cells. The immunoperoxidase detection technique was used. Culture plates were scanned by light microscopy at 100× total magnification. Positive reactions were noted in two distinct types of cells.

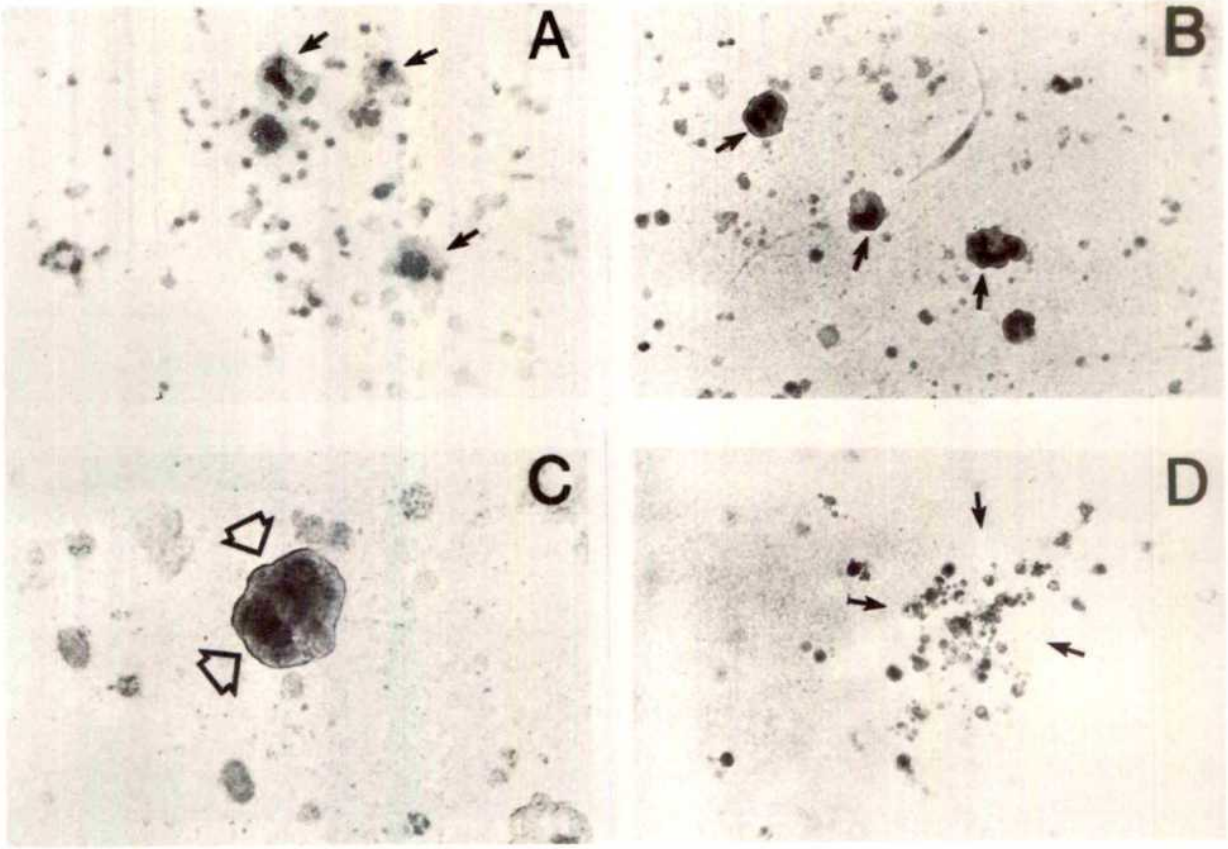

Fig 7. Expression of FV by human megakaryocytes cloned from their colony-forming unit in NU-SERUM containing plasma clot cultures immunodepleted of FV. Light-density (<1.077 gm/mL) bone marrow mononuclear cells were cloned in plasma clots for 12 days in 5% CO_2 at 37 °C. Fixed, dried clots were probed with normal rabbit serum (A) or rabbit antihuman FV antiserum (B) in conjunction with the immunoperoxidase detection technique. Large megakaryocytes with typical multilobed nuclei and generous cytoplasm are indicated by the arrows. Note the darkness of the cytoplasm and membrane rings present in large cells shown in B in comparison with similar cells shown in (A). (C) shows further enlargement of the topmost positive cell in (B). The open arrows draw attention to the dark membrane ring not present on any of the surrounding cells. The megakaryocyte measured ~30 μm in diameter. (D) is a granulocyte colony from the same culture as the cells photographed in (B). These cells are unlabeled.

One type of positive cell possessed a nonrefractile, densely granular cytoplasm. These cells grew in tight clusters and were subsequently identified as being eosinophils by Wright-Giemsa staining (data not shown). Further studies revealed that such cells were brown stained even in the absence of primary antibody because of an inability to quench their endogenous peroxidase activity. Accordingly, label accumulation in eosinophils was judged to be nonspecific, and they were excluded from further analysis.

The other type of positive cell was quite large in comparison with other cells in the cultures. In addition, such cells had multilobed nuclei and grew in colonies of loosely clustered cells (Figs 7A, 7B, 7C). By morphologic criteria, they were typical of the megakaryocyte colonies that normally develop in plasma clot cultures.[17] Their identity as cells belonging to the megakaryocyte lineage was confirmed by demonstrating that all cells of the type described labeled intensely with antiplatelet glycoprotein antiserum (data not shown). We noted, however, that not all of these cells were FV-positive. In fact, of 101 megakaryocyte colonies identified, 60 were noted to contain no FV-positive cells.

The demonstration of FV in cells cultured in an environment almost totally devoid of this protein suggested that these cells have been synthesizing FV. We therefore examined small cells contained within the colonies expressing FV to determine at what level of megakaryocyte maturation synthesis of this protein began. In contrast to our finding that ~70% of putative megakaryocyte precursor cells in bone marrow were FV-positive (Fig 5), no small cells present in the FV-expressive colonies contained convincing amounts of immunochemically detectable FV.

Factor V was not detected in any other hematopoietic cell colonies present in the cultures.

Synthesis of FV by elutriation-enriched human megakaryocytes. To document synthesis of FV by human megakaryocytes, elutriation-enriched megakaryocyte preparations (~40% pure) and an equivalent number of megakaryocyte-poor leukocytes derived from the final Percoll separation were suspended in serum-free, methionine-free DMEM to which was added 500 μCi of ^{35}S-methionine. The cell-free conditioned medium and lysates prepared from the radiolabeled cells were then applied to a FV immunoaffinity column using the B38 antibody. Material eluted from the column was then analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography (Fig 8). There was no band indicating FV production by cell in the megakaryocyte-depleted leukocyte sample (lane 1) nor in the medium in which these cells were incubated (data not shown). In contrast, lane 2 of Fig 8 reveals that lysates of the megakaryocyte-rich preparation did contain newly synthesized protein corresponding to the heavy and light chain of thrombin-activated FV, which is shown in lane 4.

The megakaryocyte-conditioned tissue culture medium (lane 3) also contained material that adhered specifically to the B38 immunoaffinity column. Three distinct bands were noted in autoradiographed material eluted from the column. One band corresponded to the light chain of FVa and another to a lower weight protein of ~54,000 mol wt, which was also observed in the thrombin-activated FV standard (lane 4). A prominent band that appeared to be somewhat heavier than the material we identified as FVa heavy chain in the megakaryocyte lysate (lane 2) and in thrombin-activated FV (lane 4) was also noted in the cell-free conditioned medium.

As expected, we did not demonstrate the activation peptide (mol wt ~150,000) in the gel since this fragment is not recognized by the B38 antibody and thus would not bind to the column. For control purposes, megakaryocyte lysate and conditioned medium were also passed over an immunoaffinity column to which had been linked purified normal mouse IgG. No bands were demonstrated in the eluate from this column in either a Coomassie-stained gel or in an autoradiograph (data not shown).

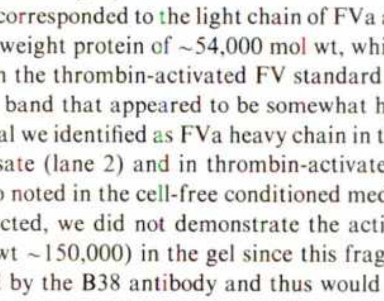

Fig 8. Biosynthesis of FV by elutriation-enriched human marrow megakaryocytes. Approximately 2 × 10⁵ megakaryocytes or ~2 × 10⁵ leukocytes were incubated in ^{35}S-methionine for 18 hours in methionine-free DMEM. Cells were lysed in the presence of proteolytic inhibitors by sonication and freeze-thawing. Lysates were clarified by centrifugation in an Epperdorf microfuge and then applied to a B38 immunoaffinity column. Nonspecifically bound material was washed from the columns, after which specifically bound protein was eluted as described. The mol wt of the bound material was determined by SDS-PAGE and autoradiography. Numbers on the left are the apparent mol wts of the indicated bands in kilodaltons (kDa). Numbers on the right with their accompanying dash marks indicate migration of known mol wt standards added to the gel. Leukocyte-derived material is shown in lane 1. Megakaryocyte-derived material is shown in lane 2. Material derived from the megakaryocyte-conditioned medium is shown in lane 3. Thrombin-activated ^{125}I-labeled FV is shown in lane 4. In lane 2 bands corresponding to the heavy (mol wt ~105,000) and light (mol wt ~74,000) chain of thrombin-activated FV (lane 4) are seen. In lane 3, a band corresponding in weight to the FVa light chain is also seen. In addition, lane 3 demonstrates a band slightly heavier than the FVa heavy chain (arrow) and another band of ~54,000 mol wt also seen in the thrombin-cleaved FV fragments (arrows) shown in lane 4. The identity of the 190-k Da band seen in lanes 1 and 2 is unknown but may represent nonspecifically bound fibronectin since it appears only in the cell lysate lanes.

DISCUSSION

Our studies of the megakaryocyte FV have proved instructive from several standpoints. First, we are now able to provide the first direct evidence that human megakaryocytes synthesize FV. Second, we have demonstrated that MoAb recognizing different epitopic domains of the FV molecule may be useful in studying the binding and synthesis of this protein by human megakaryocytes. Third, by using both bone marrow– and tissue culture–derived megakaryocytes, we have been able to examine the expression of FV as a function of cell maturation. An enhanced appreciation of megakaryocyte and FV biology has therefore been obtained.

Synthesis of proteins corresponding to the heavy and light chain of FVa was demonstrated using radiolabeled cell lysates prepared from elutriation-enriched bone marrow megakaryocytes (Fig 8, lane 2). Three other protein bands were identified in the megakaryocyte-conditioned medium (Fig 8, lane 3). One band comigrated with the known FVa light chain (Fig 8, lane 4). A smaller cleavage product was also noted that appeared to comigrate with a known FVa fragment (mol wt ~54,000) seen in lane 4 of Fig 8. This proteolytic fragment appears to represent a low–molecular weight domain of human FV recently described by Dahlback.[28] The third band noted in this preparation migrated more slowly in the gel than did the FVa heavy chain. We believe that this band may represent the result of the cleavage of a megakaryocyte α-granule FV by a protease present in the cell's cytoplasm. The α-granule FV could have come into contact with this protease during its exportation from the cell or as a result of cytolysis. A serine protease yielding different FV cleavage products than those observed with thrombin activation has been described by Kane et al[30] in the platelet cytosol. Such a protease would likely be present in mature megakaryocytes. Whether any of the putative FV cleavage products we identified is similar to the functionally distinct platelet FV described by Tracy et al[16] is a matter of speculation.

Ideally, intact FV would also have been demonstrated in the labeled cell preparations, but the extreme sensitivity of human FV to proteolysis[28] may have prevented this from being accomplished. Thus, despite the fact that numerous proteolytic inhibitors were used in the cell-lysing buffer, their onset of action may not have been rapid enough to prevent degradation of the protein. Nevertheless, our results suggest that human megakaryocytes not only synthesize FV but may actively transport or secrete this protein into their environment. Further, it appears that the FV may be secreted in an already processed form resembling, but not identical to, thrombin-activated FV.

Our data also imply that synthesis of FV by human hematopoietic cells is restricted to megakaryocytes. Tracy and co-workers have previously reported that human monocytes have detectable FV activity that appeared to be intracellular and not membrane bound.[31] Our inability to detect FV in granulocyte-macrophage colonies cloned in FV-depleted medium or to demonstrate FV in lysates of bone marrow leukocytes depleted of megakaryocytes suggests that the FV associated with monocytes may not be synthesized by these cells. Conceivably, these phagocytic cells could imbibe the protein in the process of ingesting platelets or megakaryocyte debris. Alternatively, the monocytes may bind plasma FV and internalize the protein.

During the course of this investigation, MoAb recognizing two different epitopic domains of the human FV molecule were developed. The use of these antibodies as probes for examining FV expression in human marrow and cultured megakaryocytes led to an unanticipated finding. When elutriation-enriched marrow megakaryocytes were probed with the B10 activation peptide (mol wt ~150,000)–specific MoAb, only 68% of the cells displayed labeling intensity greater than that observed when cells were labeled with the control mouse ascities (Fig 6). Since it is known that FV binding to cell surfaces is likely mediated via the light chain[32] and since the activation peptide does not bind to platelets,[10,32] nor presumably megakaryocytes, our data suggest that up to one third of marrow megakaryocytes may contain no intrinsic, ie, synthesized, FV. Accordingly, the ability to synthesize this protein may be a constitutive property that is not universally expressed by all cells of this lineage.

The in vitro culture data support the hypothesis that FV synthetic ability is restricted in some manner since only 40% of the megakaryocyte colonies contained FV-positive cells. Since the megakaryocytes were cloned in FV-deficient medium, we presume that the FV detected in these cells was synthesized and not bound. If the positive results seen in the cultures were due to a concentration and /or binding of the small amounts of FV present in the medium, then most, if not all, megakaryocytes present in the cultures should have been labeled. This supposition follows from our demonstration that the ability to bind FV is nearly universal in marrow megakaryocytes and megakaryocyte maturation in plasma clots appears to be essentially identical to maturation in vivo.[33] Absence or diminution of constitutive megakaryocyte FV synthesis or dominance of a clone or megakaryocytes incapable of synthesizing FV could equally explain the development of a familial bleeding disorder like FV Quebec.[16] Using the MoAb and in vitro culture techniques described in this study could further elucidate the pathogenesis of this and other bleeding disorders where either diminished FV synthesis or defective FV binding to cells [34] is suspected of being of etiologic importance.

These studies have also generated new information about FV expression as a function of megakaryocyte maturation. In the bone marrow smears examined, we found that both morphologically recognizable and most immature, nonmorphologically recognizable megakaryocytes expressed FV. However, in the FV-depleted plasma clot cultures, small cells in the megakaryocyte colonies were inconclusively or totally unlabeled. These data are in accord with those recently published by Vinci et al[35] who showed that other megakaryocyte α-granule proteins such as VIII:RAg and platelet factor 4 are poorly expressed in early megakaryocytes. Therefore, it appears likely that the megakaryocyte's ability to bind FV occurs early in the cell's development. In contrast, actual synthesis of this protein may not begin until later developmental stages. FV might then serve as a functional marker of megakaryocyte maturation.

Since the majority, but not all, megakaryocytes appear to synthesize factor V, it seems probable that factors other than level of cell maturation likely play a role in regulating the synthesis of this critical coagulation cofactor. Use of the tools and techniques described in this report may allow these regulatory phenomena to be elucidated.

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REFERENCES

1. Mann KG, Nesheim ME, Hibbard LS, Tracy PB: The role of Factor V in the assembly of the prothrombinase complex. Ann NY Acad Sci 370:378, 1985

2. Tracy PB, Nesheim ME, Mann KG: Coordinate binding of FVa and factor Xa to the unstimulated platelet. J Biol Chem 256:743, 1981

3. Bloom JW, Nesheim ME, Mann KG: Phospholipid-binding properties of bovine FV and FVa. Biochemistry 18:4419, 1979

4. Miletich JP, Jackson CM, Majerus PW: Properties of the factor Xa binding site on human platelets. J Biol Chem 253:6908, 1978

5. Salem HH, Bronze GJ, Miletich JP, Majerus PW: Human coagulation FVa is a cofactor for the activation of protein C. Proc Natl Acad Sci USA 80:1584, 1983

6. Maruyama I, Salems HH, Majerus PW: Coagulation FVa binds to human umbilical vein endothelial cells and accelerates protein C activation. J Clin Invest 74:224, 1982

7. Marlar RA, Kleiss AJ, Griffin JH: Human protein C: Inactivation of the activated molecule. Ann NY Acad Sci 370:303, 1981

8. Gewirtz A, Keefer M, Memoli M, Hansel BC, Chiu HC, Colman RW: Evidence for synthesis of coagulation Factor V by human megakaryocytes. Clin Res 32:307, 1984

9. Nichols NL, Gastineau DA, Solberg EA, Mann KG: Identification of human megakaryocyte coagulation factor V. Blood 65:1396, 1985

10. Kane WH, Majerus PW: The interaction of human coagulation FVa with platelets. J Clin Invest 257:3963, 1982

11. Tracy PB, Eide LL, Bowie EJW, Mann KG: Radioimmunoassay of FV in human plasma and platelets. Blood 60:59, 1982

12. Chiu HC, Schick PK, Colman RW: Biosynthesis of Factor V in isolated guinea pig megakaryocytes. J Clin Invest 75:339, 1985

13. Nachman RL, Levine R, Jaffe EA: Synthesis of FVIII antigen by cultured guinea pig megakaryocytes. J Clin Invest 60:914, 1977.

14. Leven RM, Schick PK, Budzynski AZ: Fibrinogen synthesis by isolated guinea pig megakaryocytes. Blood 65:501, 1985

15. Chesney CM, Pifer D, Colman RW: Subcellular localization and secretion of factor V from human platelets. Proc Natl Acad Sci USA 78:5080, 1981

16. Tracy PB, Giles AR, Mann KG, Eide LL, Hoogerdoorn H,

Rivard GE: Factor V (Quebec): a bleeding diathesis associated with a qualitative platelet FV deficiency. J Clin Invest 74:1221, 1984

17. Mazur EM, Hoffman R, Chasis J, Marchesi S, Bruno E: Immunofluorescent identification of human megakaryocyte colonies using an anti-platelet glycoprotein antiserum. Blood 57:277, 1981

18. Berkow RL, Straneva JE, Bruno E, Beyer GS, Burgess JS, Hoffman R: Isolation of human megakaryocytes by density centrifugation and counterflow centrifugal elutriation. J Lab Clin Med 103:811, 1984

19. Levine RE, Fedorko ME: Isolation of intact megakaryocytes from guinea pig femoral marrow. J Cell Biol 69:159, 1976

20. Skoog WA, Beck WS: Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Blood 11:436, 1956

21. Mazur EM, Hoffman R, Bruno E: Regulation of human megakaryocytopoiesis. An in vitro analysis. J Clin Invest 68:733, 1981

22. Gewirtz AM, Bruno E, Elwell J, Hoffman R: In vitro studies of megakaryocytopoiesis in thrombocytotic disorders of man. Blood 61:834, 1983

23. Chiu HC, Whitaker WE, Colman RW: Heterogeneity of human FV deficiency. Evidence for the existence of antigen-positive variants. J Clin Invest 72:493, 1983

24. Marchesi SL, Chasis JA: Isolation of human platelet glycoprotein. Biochim Biophys Acta 555:442, 1979

25. Ware AG, Murphy RC, Seegers WH: The function of Ac-globulin. Science 106:618, 1947

26. Fraker PC, Speck SC Jr: Protein and cell membrane iodinations with a sparingly soluble chloramide 1, 3, 4, 6-Tetrachloro-3α 6α diphenylgly Coluril. Biochem Biophys Res Commun 80:849, 1978

27. Laemmli UK: Cleavage of the structural proteins during assembly at the head of the bacteriophage T4. Nature 277:680, 1970

28. Dahlback B: Ultrastructure of human coagulation FV. J Biol Chem 250:1347, 1985

29. Long MW, Williams N: Immature megakaryocytes in the mouse: Morphology and quantitation by acetylcholinesterase staining. Blood 58:1032, 1981

30. Kane WH, Mruk JS, Majerus PW: Activation of coagulation factor V by a platelet protease. J Clin Invest 70:1092, 1982

31. Tracy PB, Rohrbach MS, Mann KG: Functional prothrombinase complex assembly on isolated monocytes and lymphocytes. J Biol Chem 258:7264, 1983

32. Pusey ML, Nelsestuen GL: Membrane binding properties of blood coagulation FV and derived peptides. Biochemistry 23:6202, 1984

33. Vainchenker W, Breton-Gorius J, Chapman J: Induction of human megakaryocyte colonies in vitro and ultrastructural aspects of the maturationmin in Evatt BL, Levine RE, and Williams NT (eds): Megakaryocyte Biology and Precursors: In vitro Cloning and Cellular Properties. New York, Elsevier, 1981, p 131

34. Miletich, JP, Kane WH, Hoffman SL, Stanford N, Majerus PW: Deficiency of Factor Xa-Factor Va binding sites on the platelets of a patient with a bleeding disorder. Blood 54:1015, 1979

35. Vinci G, Tabilio A, Deschamps JF, VanHaeke D, Henri A, Guichard J, Tetterov P, Lansdorp PM, Herrend T, Vainchenker W, Breton-Gorius J: Immunological study of in vitro maturation of human megakaryocytes. Br J Haematol 56:589, 1984

Platelets Modulate the Proteolysis of Factor VIII:C Protein by Plasmin

By Margaret E. Rick and Dennis M. Krizek

Factor VIII coagulant protein (VIII:C) functions as a critical cofactor with factor IXa, calcium ions, and phospholipid during the activation of factor X. In the course of this reaction, the activity of VIII:C is first increased and then is destroyed by one or more serine proteases that are part of the coagulation sequence. In this study, we have investigated the influence of platelets on the inactivation of VIII:C by plasmin. Platelets were separated from plasma proteins in the presence of granule release inhibitors and were incubated with plasmin and isolated VIII:C or the complex of purified VIII:C/von Willebrand factor (vWF); VIII:C activity and antigen levels were assessed over time. In the presence of platelets, the isolated VIII:C showed an initial increase in VIII:C activity that was not present when platelets were absent, and the VIII:C/vWF showed an increase in VIII:C activity over that seen when platelets were absent. In addition, platelets stabilized VIII:C activity over a one-hour time course when compared with buffer. The VIII:C antigen did not increase and decreased slowly whether platelets were present or absent. Preincubating the platelets with ristocetin, collagen, or plasmin did not alter the results, and experiments using platelets from a patient with severe von Willebrand's disease also showed a pattern similar to that seen with normal platelets. Experiments using fixed platelets or phospholipid vesicles showed that they did not support the activation reaction or delay the inactivation reaction. These studies demonstrate that platelets modulate the activation and inactivation of VIII:C by plasmin, apparently by a mechanism that is independent of the platelet release reaction.

FACTOR VIII coagulant protein (VIII:C) is a labile clotting factor that circulates in plasma as part of a noncovalent complex with von Willebrand factor (vWF).[1] VIII:C functions as a cofactor with factor IXa, Ca^{++} and phospholipid in the activation of factor X, and during the clotting process it is degraded and its activity is decreased.[2] VIII:C is notoriously susceptible to activation and/or degradation by several serine proteases, including those that are part of the coagulation sequence[3-10]; plasmin is one of the better characterized proteases that degrades VIII:C, and it is suitably located to function within the forming blood clot at the platelet surface where VIII:C and other coagulation factors are also localized.

Recent studies have suggested that phospholipids may interact with VIII:C in a noncovalent association that protects VIII:C from specific antibodies directed against the protein.[11] Platelet membranes may also potentially protect VIII:C from the action of proteolytic enzymes by a similar mechanism. The current studies examine the influence of intact platelets on the interaction of VIII:C with plasmin. They show that platelets not only slow the loss of VIII:C activity caused by plasmin but also enhance the initial activation of VIII:C activity that is observed with low concentrations of plasmin.[11] A similar pattern is seen with both isolated VIII:C and the VIII:C/vWF complex.

MATERIALS AND METHODS

Plasmin and aprotinin were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind). The plasmin was purified by affinity chromatography and had a specific activity of 8 U/mg. Soybean trypsin inhibitor (SBTI), diisopropylfluorophosphate (DFP), procaine HCl, 2-chloroadenosine, rabbit brain cephalin, and phosphatidyl serine (bovine brain) were obtained from Sigma Chemical Co (St Louis), and D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone (P-PACK) from Calbiochem-Behring Corp (La Jolla, Calif). Lecithin (phosphatidyl choline) was obtained from P-L Biochemicals (Milwaukee). Collagen (calf skin) was obtained from Worthington Biochemicals (Freehold, NJ) and bovine serum albumin (BSA) from Baker Chemical Co (Phillipsburg, NJ). Factor VIII–deficient substrate and factor VIII inhibitor plasma (for inhibition studies) were obtained from George King Biomedical (Overland Park, Kan), and the β-thromboglobulin (β-TG) radioimmunoassay kit was obtained from Amersham Corp (Arlington Heights, Ill). Monoclonal antibodies to glycoprotein Ib (GPIb) and GPIIb/IIIa were the gift of Dr Barry S. Coller (SUNY, Stony Brook, NY) and the fluoresceinated goat antimouse IgG was obtained from Becton Dickinson (Sunnyvale, Calif). All other reagents were of reagent grade or higher and were commercially available.

VIII:C preparations. Two preparations of VIII:C were used: VIII:C purified as part of a complex with vWF (VIII:C/vWF) and isolated VIII:C that was separated from vWF and other plasma proteins. The VIII:C/vWF was purified from commercial concentrates (Hyland Laboratories, Costa Mesa, Calif) as previously reported[12] with the following modifications: the protease inhibitors, SBTI (0.1 mg/mL), aprotinin (10 U/mL) and P-PACK (10^{-5} mol/L) were added during the initial dissolution of the factor VIII concentrate and were added to the buffers at all steps until resuspension of the 12% polyethylene glycol 4000 precipitate. The 6% agarose column was eluted with veronal-buffered saline (VBS) containing aprotinin, 10 U/mL. Typical preparations contained 15 to 20 U/mL VIII:C activity, 22 to 28 U/mL VIII:CAg, and 45 to 55 U/mL VIII R:Ag; the protein concentration was 0.7 to 1.0 mg/mL. Assessment by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (5%) showed that a major single band of 220,000 daltons was visible after the reduction of disulfide bonds and that less than 8% of the protein was seen as bands of lower molecular weight. The isolated VIII:C was prepared as reported from plasma with the use of a solid-phase antibody to vWF.[12] Modifications included the use of fresh-frozen heparinized plasma (5 U/mL) instead of citrated plasma, the addition of 2 mmol/L DFP and 100 U/mL aprotinin to the plasma before freezing and a second addition of 2 mmol/L DFP during thawing, and the inclusion of 2 mmol/L DFP in the equilibrating buffer for the affinity column and the wash and eluting buffers. Column fractions containing VIII:C were not concentrated but were dialyzed against VBS/2 mmol/L DFP/2 mmol/L Ca^{++} and then VBS/2 mmol/L Ca^{++} on the day of use. Typical prepara-

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tions contained 0.5 to 1.5 U/mL VIII:C activity, 1.0 to 2.2 U/mL VIII:CAg, and <0.002 U/mL VIII R:Ag.

Preparation of platelets. Blood was collected in a 1/20 vol of an anticoagulant inhibitor solution: this included $MgCl_2$ (25 mmol/L), procaine HCl (15 mmol/L), 2-chloroadenosine (0.05 mmol/L), and $CaCl_2$ (2 mmol/L), all given as final concentrations. Platelet-rich plasma (PRP) was prepared by centrifugation at 600 g for three minutes at room temperature; the PRP was removed and Tyrode's buffer was added to the remaining cells, which were gently mixed and then centrifuged at 600 g for three minutes. This wash was removed from the cells and combined with the original PRP, and the mixture was layered on a 10% to 20% discontinuous arabinogalactan gradient[13] that was centrifuged for 30 minutes at 2000 g at room temperature. The Tyrode's wash and the arabinogalactan solutions both contained the anticoagulant-inhibitor mixture previously noted. After centrifugation, the platelet layer was cut from the tube, 8 ml of Tyrode's buffer with 0.5% BSA was added, and the platelets were pelleted by centrifugation for ten minutes at 2000 g. The platelets were resuspended in Tyrode's/0.5% BSA to give the desired final concentration and were used within two hours. In several experiments, either ristocetin (0.5 mg/mL final concentration), collagen (30 μg/mL final concentration), or plasmin (0.007 U/mL final concentration) were added to the platelets after separation on the arabinogalactan gradient. After a five-minute incubation at 37 °C, the platelets were washed with 10 mL of Tyrode's/0.5% BSA (including aprotinin, 0.3 mg/mL, in the wash for the incubation containing plasmin) and resuspended in Tyrode's/0.5% BSA to give the desired final concentration. The same procedure was used to isolate platelets from a patient with severe von Willebrand's disease (VIII:C, 0.04 U/mL; VIII R:Ag, <0.002 U/mL; ristocetin cofactor, <0.03 U/mL; platelets contain undetectable VIII R:Ag). In experiments using formalin-fixed platelets, the preparation was carried out as previously noted through the arabinogalactan step; the platelet layer from the gradient was then suspended in 50 mL of 1.1% paraformaldehyde diluted in Tyrode's (without BSA) and incubated at 37 °C for 15 minutes and at 4 °C for 15 minutes with occasional mixing. The platelets were spun at 2000 g for ten minutes and washed twice with Tyrode's/0.5% BSA buffer.

Incubation mixtures. The incubations were carried out as follows: the VIII:C preparation (0.5 to 1.5 U/mL) and washed platelets (200,000 to 400,000/μL) were added to a polypropylene tube in a total volume of 400 μL and gently mixed at 37 °C; a subsample was removed for VIII:C and VIII:CAg assays. One one-hundreth volume of plasmin (0.002 to 0.007 U/mL final concentration) was added, and subsamples were removed at the indicated time points for immediate VIII:C activity assay and for VIII:CAg assay. Samples for VIII:CAg assay were immediately diluted in borate saline (B/S) containing 0.34 mg/mL aprotinin and were snap-frozen at −70 °C for up to five days prior to assay. With each experiment, control incubations of VIII:C + plasmin + buffer, VIII:C + buffer + platelets, and buffer + platelets + plasmin were included.

In experiments using phospholipids, a similar volume of phospholipid was added in place of the platelets in the incubation mixture. Phospholipid vesicles were prepared from phosphatidyl choline (PC) (25 mg) and phosphatidyl serine (PS) (8 mg) using modifications of the method of Nesheim et al[14] and were used at concentrations of approximately 200 μmol/L.

To evaluate the potential effects of plasmin on the release of α-granules by the isolated platelets, a standard incubation mixture of the aforementioned concentrations of VIII:C/vWF, platelets, and plasmin (0.007 U/mL) was prepared at 37 °C; samples were removed at indicated time points and centrifuged at room temperature for ten minutes at 15,000 g and the supernatant removed. The platelet pellet was frozen and thawed five times, and subsequently, the pellets and supernatants were assayed by radioimmunoassays for β-TG and VIII R:Ag.[15] To assess plasmin effects on representative glycoproteins in the platelet membrane, a similar incubation mixture containing VIII:C/vWF, platelets, and plasmin (0.007 U/mL) or buffer control was prepared at 37 °C. Samples containing 10^8 platelets were removed before plasmin (or buffer) addition and at various time points after plasmin addition, diluted with an equal volume of 2% paraformaldehyde/aprotinin (10 μg/mL), and incubated at 37 °C for 15 minutes. Ten milliliters of Tyrode's buffer (without Ca^{++}) containing 10 mmol/L EDTA and 1% BSA were then added to each tube; the tubes were gently mixed and spun at 2000 g for seven minutes. After aspiration of the supernatant, platelets were incubated with a monoclonal antibody to GPIb (0.06 μg/mL final concentration) or GPIIb/IIIa (0.10 μg/mL final concentration) for 30 minutes, washed with Tyrode's/10 mmol/L EDTA/1% BSA, and incubated with a goat antimouse IgG fluorescent antibody. After washing again, the fluorescence of each sample was evaluated by flow cytometry with an EPICS V cytofluorometer using a coherent argon laser.[16]

Assays. VIII:C activity was measured using a one-stage assay based on the activated partial thromboplastin time (aPTT), and VIII:CAg and VIII R:Ag were measured by radioimmunoassays.[15,17] The VIII:CAg assay uses an antibody purified from a hemophiliac with a high-titer inhibitor.

In experiments to demonstrate the specificity of the apparent VIII:C activity during plasmin activation, subsamples from an incubation mixture (see the previous methods) were diluted into B/S containing a concentrated IgG fraction from an VIII:C inhibitor plasma. The IgG (38 mg/mL) was prepared from citrated plasma by caprylic acid precipitation.[18] The subsamples were allowed to incubate at 37 °C for two minutes, a final dilution was made into VBS, and the VIII:C activity assay was performed immediately. In a parallel experiment, the subsamples were diluted into B/S containing nonimmune IgG, incubated, and then further diluted in VBS prior to VIII:C activity assay.

Buffers included VBS (0.125 mol/L NaCl, 0.015 mol/L barbital, and 0.01 mol/L sodium barbital, pH 7.5), B/S (0.036 mol/L boric acid, 0.005 mol/L NaOH and 0.159 mol/L NaCl, pH 7.8) and Tyrode's (0.136 mol/L NaCl, 2 mmol/L KCl, 1 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 0.9 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, and 1 g/L glucose, pH 7.4).

RESULTS

Experiments to assess the potential effects of plasmin (0.007 U/mL) on the release reaction of our isolated platelet preparations are shown in Table I. There is no progressive release of α-granule constituents after plasmin addition as measured by the release of VIII R:Ag or β-TG, and the major fraction of these platelet constituents remains with the platelet pellets. Plasmin at this concentration also does not grossly affect the antigenic reactivity of two platelet membrane glycoproteins during the one-hour incubation time as assessed by the binding of monoclonal antibodies to GPIb and GPIIb/IIIa. We were unable to detect any change in the fluorescence signal after incubation with plasmin (0.007 U/mL) for one hour using an indirect immunofluorescent method with detection by flow cytometry. It thus appears that the changes to be reported here are due primarily to the action of plasmin on VIII:C and to possible effects on platelet membrane phospholipids.

When isolated VIII:C was incubated with plasmin in the presence of physiologic numbers of platelets, the VIII:C activity initially increased threefold and then slowly declined

Table 1. α-Granule Release Proteins

	VIII R:Ag		β-TG	
	Platelet	SN	Platelet	SN
Preplasmin	0.030	<0.002	41.8	19.9%
0.5 (min)	0.033	<0.002	55.6	20.1%
3	0.024	<0.002	44.4	19.8%
6	0.020	<0.002	38.9	23.7%
10	0.017	<0.002	45.1	22.7%
20	0.020	<0.002	48.3	13.2%
30	0.026	<0.002	51.9	16.1%
60	0.018	<0.002	50.9	16.3%

Platelet pellets and supernatants (SN) were assayed for VIII R:Ag and β-TG before (pre) addition of 0.007 U/mL plasmin and at varying time points thereafter (see Materials and Methods). Values for VIII R:Ag are expressed as units/2 × 10^8 platelets and in the SN as the amount released from 2 × 10^8 platelets. Values for β-TG are expressed as ng/2 × 10^6 platelets and as the percentage in the SN from 2 × 10^6 platelets.

to near baseline by 60 minutes (Fig 1A, n = 6). If platelets were not present, the VIII:C activity did not increase, but decreased by 65% throughout a one-hour incubation period (Fig 1A); control tubes containing buffer or platelets and VIII:C (no plasmin) retained >75% of the initial activity for the same time period. Also, the control incubation of platelets and plasmin (no VIII:C) did not generate any factor VIII–like activity in the VIII:C assay. A similar pattern was seen with the VIII:C/vWF preparation (Figure 1B, n = 7) except that the increase observed with platelets was not quantitatively as great and a small increase in VIII:C activity was seen in the absence of platelets. Control incubations containing buffer or platelets and VIII:C retained >80% of their initial activity at one hour. In the experiments using platelets that had been incubated with ristocetin (n = 5), collagen (n = 4), or plasmin (n = 4) prior to incubation with VIII:C or VIII:C/vWF, patterns similar to those seen with fresh platelets were observed (data not shown).

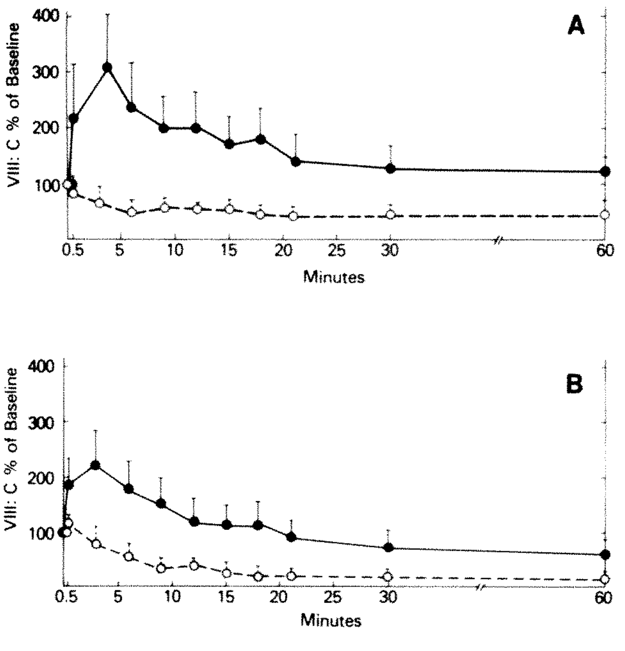

Fig 1. (A) The effect of plasmin (0.007 U/mL) on VIII:C activity in the isolated VIII:C preparation (0.52 to 0.98 U/mL) in the presence or absence of platelets (200,000 to 400,000/μL, n = 6). ●—VIII:C + Plasmin + Platelets; O---VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:C activity in the VIII:C/vWF preparation (0.68 to 1.2 U/mL) in the presence or absence of platelets (200,000 to 400,000/μL, n = 7). Bars indicate standard error. ●—VIII:C/vWF + Plasmin + Platelets; O--VIII:C/vWF + Plasmin + Buffer.

The specificity of the observed increase in VIII:C activity was demonstrated in an experiment in which the samples from the incubation mixture were diluted in a buffer containing anti-VIII:C antibody (see Materials and Methods). The shortening of the coagulation times was abolished by the incubation of samples with antibody, indicating that the observed shortening in the clotting times was indeed due to VIII:C activity (Table 2).

VIII:CAg values decreased slightly to 65% to 70% of the baseline during the incubation of isolated VIII:C with this concentration of plasmin, either in the presence or absence of platelets (Fig 2A). No increase in VIII:CAg was observed. Control incubations with buffer or platelets (no plasmin) showed that the VIII:CAg was stable throughout this time period (>80% of the baseline). Similar values were seen in the VIII:C/vWF preparation except that VIII:CAg values declined further (40% of the baseline) when platelets were absent from the incubation mixture (Fig 2B).

In experiments using platelets isolated from a patient with severe von Willebrand's disease (Figs 3A and 3B, n = 3 for each factor VIII preparation), the VIII:C activity in each factor VIII preparation increased and slowly declined in a pattern like that seen with normal platelets. The VIII:CAg values (Figs 4A and 4B) were also similar to those observed with normal platelets.

When fixed platelets were used in place of fresh platelets, they did not promote the activation of VIII:C by plasmin when incubated with isolated VIII:C (n = 4), and they supported only a very modest elevation in the VIII:C/vWF preparation (Fig 5, n = 8). Phospholipid vesicles failed to support activation in either preparation.

DISCUSSION

Our studies indicate that platelets modulate the effects of plasmin on VIII:C, enhancing the activation of VIII:C

Table 2. Specificity of VIII:C Activity After Addition of Plasmin

	VIII:C Activity	
Incubation Time	Nonimmune	Anti-VIII:C
0 min (preplasmin)	1.20 U/mL*	1.15 U/mL*
2 min	1.50	<0.002
5 min	1.75	<0.002
8 min	0.56	<0.002

*Preplasmin samples were diluted into nonimmune IgG to obtain baseline VIII:C values.

Timed samples from an incubation mixture of VIII:C, platelets, and plasmin were diluted in buffer containing normal (nonimmune) IgG or anti-VIII:C IgG (see Materials and Methods). After a further two-minute incubation at 37 °C, an VIII:C activity assay was performed.

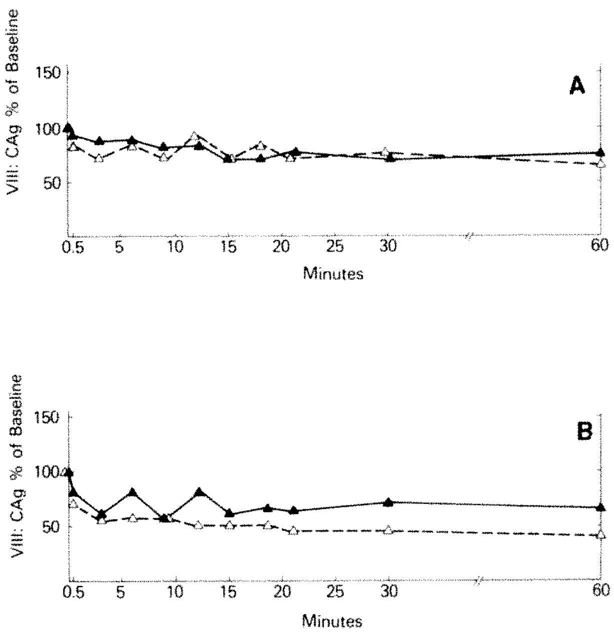

Fig 2. (A) The effect of plasmin (0.007 U/mL) on VIII:CAg in the isolated VIII:C preparation in the presence or absence of platelets, n = 6. ▲—VIII:C + Plasmin + Platelets; △---VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:CAg in the VIII:C/vWF preparation in the presence or absence of platelets, n = 7. ▲—VIII:C/vWF + Plasmin + Platelets; △--VIII:C/vWF + Plasmin + Buffer.

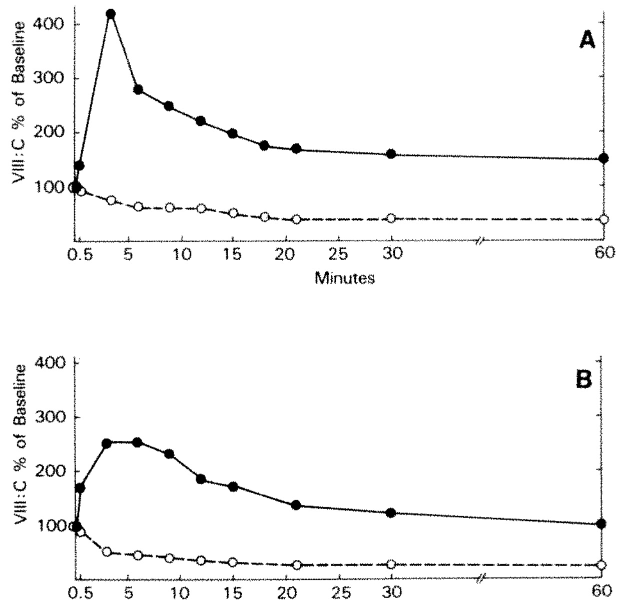

Fig 3. (A) The effect of plasmin (0.007 U/mL) on VIII:C activity in the isolated VIII:C preparation (0.62 to 0.78 U/mL) in the presence or absence of severe von Willebrand's disease (vWD) platelets (200,000 to 400,000/μL, n = 3). ●—VIII:C + Plasmin + Platelets; ○--VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:C activity in the VIII:C/vWF preparation (0.65 to 0.96 U/mL) in the presence or absence of severe vWD platelets, n = 3. ●—VIII:C/vWF + Plasmin + Platelets; ○--VIII:C/vWF + Plasmin + Buffer.

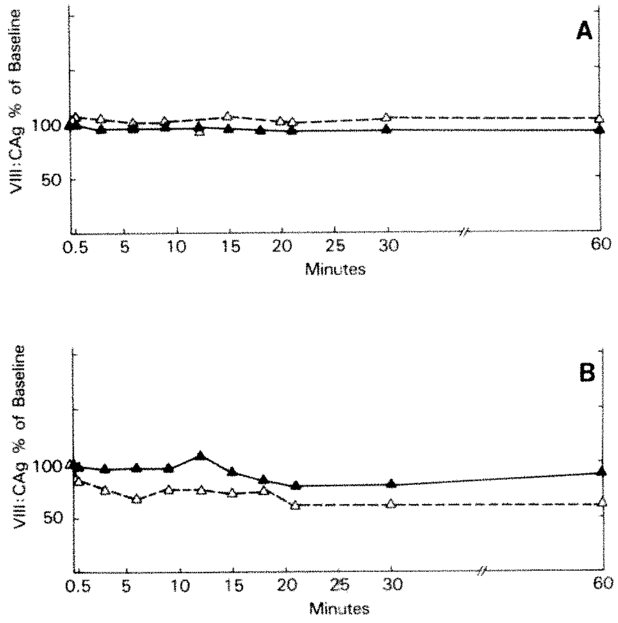

Fig 4. (A) The effect of plasmin (0.007 U/mL) on VIII:CAg in the isolated VIII:C preparation in the presence or absence of severe vWD platelets, n = 3. ▲—VIII:C + Plasmin + Platelets; △--VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:CAg in the VIII:C/vWF preparation in the presence or absence of severe vWD platelets, n = 3. ▲—VIII:C/vWF + Plasmin + Platelets; △--VIII:C/vWF + Plasmin + Buffer.

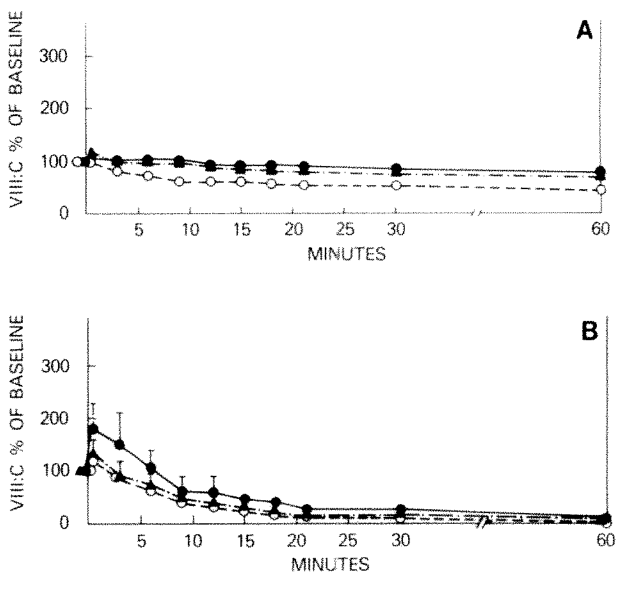

Fig 5. (A) The effect of plasmin (0.007 U/mL) on VIII:C activity in the isolated VIII:C preparation (0.49 to 0.83 U/mL) in the presence of fixed platelets, phospholipid vesicles, or buffer, n = 4 each for fixed platelets or phospholipid vesicles. ● VIII:C + Plasmin + Fixed Platelets; ▲ VIII:C + Plasmin + PL Vesicles; ○ VIII:C + Plasmin + Buffer. (B) The effect of plasmin (0.007 U/mL) on VIII:C activity in the VIII:C/vWF preparation (0.70 to 1.1 U/mL) in the presence of fixed platelets, phospholipid vesicles, or buffer. n = 8 each for fixed platelets or phospholipid vesicles. Bars indicate standard error. ● VIII:C/vWF + Plasmin + Fixed Platelets; ▲ VIII:C/vWF + Plasmin + PL Vesicles; ○ VIII:C/vWF + Plasmin + Buffer.

activity that occurs with low concentrations of plasmin. We have previously reported a small increase in VIII:C activity after incubation of VIII:C/vWF with plasmin (no platelets present), but we did not observe any increase in activity after incubation of an isolated VIII:C preparation with plasmin.[10] Other studies that have generally used higher plasmin concentrations in the absence of platelets or phospholipids have shown only a decrease in VIII:C activity following incubation of human VIII:C/vWF with plasmin[3,4,6-9]; bovine VIII:C/vWF, however, was reported to be activated and then degraded by plasmin.[5]

The activation of VIII:C by plasmin when platelets are present appears greater in the isolated VIII:C preparation than in the VIII:C/vWF. Although an absolute quantitative comparison cannot be made because of the different methods of purification, each preparation had similar VIII:C activity and VIII:CAg values, and the greater activation was seen consistently with the isolated VIII:C ($n = 6$). This implies that the conformation of VIII:C may be altered in a different way by platelets depending on whether VIII:C is complexed with vWF.

We did not observe any increase in VIII:CAg in either of the VIII:C preparations. The loss of VIII:CAg was more rapid in the VIII:C/vWF preparation than in the isolated VIII:C, an observation previously noted when platelets were not present.[10] It is possible that the conformation of VIII:C is altered when it is complexed with vWF such that the interaction with plasmin is optimized.

The mechanism by which platelets enhance VIII:C activation and slow the degradation by plasmin does not appear to involve the platelet release reaction. Although plasmin at higher concentrations can activate platelets, causing aggregation and release,[19] we found no progressive release of α-granule proteins under the conditions used in these experiments (Table 1). In addition, preincubating the platelets with plasmin before their incubation with factor VIII (and additional plasmin) did not cause any change in the activation pattern when compared with fresh washed platelets. The observation that fixed platelets do not support the activation as well as fresh platelets (Fig 5) suggests that there may be a platelet membrane alteration that plays a role in the activation reaction that cannot occur after fixation of the platelets.

We examined the possibility that platelet vWF that is present at the platelet surface might serve as the important platelet membrane component in this reaction by using platelets from a patient with severe von Willebrand's disease. Despite the lack of measurable vWF in these platelets, they supported the activation reaction by plasmin as well as normal platelets did. Additionally, the similar activation patterns seen using platelets that had been preincubated with either ristocetin or collagen suggests that exposure of GPIb or GPIIb/IIIa is not responsible for these effects.

Initial studies to examine the role of phospholipid in this reaction used phospholipid vesicles composed of PC:PS in a 3:1 ratio; they did not support the activation of VIII:C by plasmin when compared with platelets (Fig 5). More studies examining the effects of phospholipid vesicles of different composition are necessary to further explore the role of specific phospholipids in this reaction.

Other studies have demonstrated the formation of a complex between VIII:C and phospholipid[20-23] and have shown a protective effect of phospholipid when human antibodies to VIII:C are incubated with VIII:C/vWF and crude phospholipid.[11] When VIII:C is bound to phospholipid, less VIII:CAg (but not VIII:C activity) can be measured,[24,25] implying that the phospholipid binding occurs at a site on VIII:C that is close to the antigen-binding site. It is also near (sterically) the active cofactor site since the VIII:C activity is partially protected from inactivation by added antibody to VIII:C.

Platelets provide a large phospholipid surface available for binding VIII:C as well as other coagulation proteins, and recent studies have shown that platelets specifically bind plasminogen and plasmin at the cell surface.[26] The current studies demonstrate that platelets modulate the activation and inactivation or degradation of VIII:C activity by plasmin. Our evidence indicates that platelet membrane alterations, independent from platelet release, may be important in the reaction. It is likely that platelets also modulate the effects of other proteolytic enzymes on VIII:C and are another important component in the regulation of the coagulation cascade at this level.

REFERENCES

1. Hoyer LW: The factor VIII complex: Structure and function. Blood 58:1, 1981

2. Davie EW, Fujikawa K: Basic mechanisms in blood coagulation. Ann Rev Biochem 44:799, 1975

3. Pasquini R, Hershgold EJ: Effects of plasmin on human factor VIII (AHF). Blood 41:105, 1973

4. Kirby EP, Martin N, Marder VJ: Degradation of bovine factor VIII by plasmin and trypsin. Blood 43:629, 1974

5. Legaz ME, Weinstein MJ, Heldebrandt CM, Davie EW: Isolation, subunit structure and proteolytic modification of bovine factor VIII: Ann NY Acad Sci 240:43, 1975

6. McKee PA, Andersen JC, Switzer ME: Molecular structural studies of human factor VIII. Ann NY Acad Sci 240:8, 1975

7. Atichartakarn V, Marder VJ, Kirby EP, Budzynski AZ: Effects of enzymatic degradation on the subunit composition and biologic properties of human factor VIII: Blood 51:281, 1978

8. Henriksson P, Nilsson IM: Effects of leukocytes, plasmin and thrombin on clotting factors. A comparative in vitro study. Thromb Res 16:301, 1979

9. Cockburn CG, de Beaufre-Apps RJ, Wilson J, Hardisty RM: Parallel destruction of factor VIII procoagulant activity and an 85,000 dalton protein in highly purified factor VIII/vWF. Thromb Res 21:295, 1981

10. Rick ME, Krizek DM: Degradation of factor VIII coagulant antigen by proteolytic enzymes. Br J Haematol 61:477, 1985

11. Barrowcliffe TW, Kemball-Cook G, Gray E: Binding to phospholipid protects factor VIII from inactivation by human antibodies. J Lab Clin Med 101:34, 1983

12. Rick ME: Activation of factor VIII by factor IXa. Blood 60:744, 1982

13. Corash L, Shafer B, Perlow M: Heterogeneity of human whole blood platelet subpopulations. II. Use of a subhuman primate model to analyze the relationship between density and platelet age. Blood 52:726, 1978

14. Nesheim ME, Taswell JB, Mann KG: The contribution of bovine factor V and factor Va to the activity of prothrombinase. J Biol Chem 254:10952, 1979

15. Hoyer LW: Immunologic studies of antihemophilic factor (AHF, factor VIII). IV. Radioimmunoassay of AHF antigen. J Lab Clin Med 80:822, 1972

16. Marti GE, Magruder L, Vail M, Gralnick HR: Assessment of platelet membrane antigens by flow cytometric techniques. (manuscript in preparation)

17. Lazarchick J, Hoyer LW: Immunoradiometric measurement of the factor VIII procoagulant antigen. J Clin Invest 62:1048, 1978

18. Steinbuch M, Audran R: The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch Biochem Biophys 134:279, 1969

19. Guccione MA, Kinlough-Rathbone RL, Packham MA, Harfenist FJ, Rand ML, Greenberg JP, Perry DW, Mustard JF: Effects of plasmin on rabbit platelets. Thromb Haemost 53:8, 1985

20. Hemker HC, Kahn MJP, Devilee PP: The adsorption of coagulation factors onto phospholipids. Its role in the reaction mechanism of blood coagulation. Thromb Diath Haemorrh 24:214, 1970

21. Andersson LO, Brown JE: Interaction of factor VIII–von Willebrand factor with phospholipid vesicles. Biochem J 200:161, 1981

22. Andersson LO, Thuy LP, Brown JE: Affinity chromatography of coagulation factors II, VIII, IX and X on matrix-bound phospholipid vesicles. Thromb Res 23:481, 1981

23. Lajmanovich A, Hudry-Clergeon G, Freyssinet JM, Marguerif G: Human factor VIII procoagulant activity and phospholipid interaction. Biochim Biophys Acta 678:132, 1981

24. Yoshioka A, Peake IR, Furlong BL, Furlong RA, Giddings JC, Blood AL: The interaction between factor VIII clotting antigen (VIIICAg) and phospholipid. Br J Haematol 55:27, 1983

25. Broden K, Brown JE, Carton C, Andersson LO: Effect of phospholipid on factor VIII coagulant activity and coagulant antigen. Thromb Res 30:651, 1983

26. Miles LA, Plow EF: Binding and activation of plasminogen on the platelet surface. J Biol Chem 260:4303, 1985

Successful Donor Cell Engraftment in a Recipient of Bone Marrow from a Cadaveric Donor

By Bruce R. Blazar, Larry C. Lasky, John P. Perentesis, Kathleen V. Watson, Stephen E. Steinberg, Alexandra H. Filipovich, Harry T. Orr, and Norma K.C. Ramsay

A 12-year-old male with acute lymphocytic leukemia received donor bone marrow from his histocompatible father whose marrow was harvested 40 minutes postmortem after he suffered a myocardial infarction. The marrow was stored in liquid nitrogen for 17 days prior to infusion into the recipient. Trypan blue viability was >99% for the fresh marrow. Progenitor cell assays revealed that 20% of the CFU-MIX, 16% of the BFU-E, 10% of the CFU-E, and 17% of the CFU-GM were spared during the cryopreservation period. Posttransplantation, the recipient had a leukocyte count $>10^3/\mu L$ by day 26. Southern blotting analysis documented the donor origin of the peripheral blood mononuclear cells and granulocytes isolated 46 days posttransplantation. Unfortunately, the patient died of complications relating to graft-*v*-host disease 67 days following transplantation. This case demonstrates the feasibility of cadaveric marrow as a source of donor cells and is the first reported case of documented leukocyte engraftment in a recipient of cadaveric marrow.

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WITH THE INCREASING number of diseases currently being treated by bone marrow transplantation (BMT) and the paucity of available histocompatible sibling donors, considerable effort is being directed toward developing strategies for the use of alternative donors. Related donors who are mismatched with the recipient at one or more HLA loci but who share at least one haplotype with the recipient have been used.[1-4] In some instances, the donor marrow has been processed in vitro to remove T lymphocytes as a prophylactic measure against graft-*v*-host disease (GVHD), whereas other donors who have minimal reactivity in a mixed lymphocyte culture (MLC) test with the recipient have been used without T cell depletion of the donor marrow.[1-14] More recently, unrelated donors who are histocompatible at class 1 and/or class 2 loci have been selected from volunteer registries, usually coordinated through local and regional blood centers.[15-22] The use of volunteer unrelated donors introduces new moral, legal, and logistic issues.[23] For example, possible adverse effects of donor cell harvesting and blood replacement, if necessary, complicate the usage of volunteer unrelated donors.[24,25]

Results of human in vitro cell culture techniques and animal bone marrow transplantation experiments with marrow from cadaveric donors have stimulated some centers to begin storage of human cadaveric marrow, with or without T cell depletion prior to freezing, for future marrow reinfusion.[26,27] The use of cadaveric donor marrow, if successful, could be an important step in providing a much expanded donor pool. Until now, there have been no reports of stable donor cell engraftment following marrow reinfusion from a cadaveric donor. In our case, donor cell engraftment was suggested by the occurrence of severe GVHD post-BMT, and the presence of donor mononuclear cells and granulocytes was documented by Southern blotting techniques. Unfortunately, the patient died 67 days post-BMT of complications related to severe GVHD. This case demonstrates the feasibility of viable marrow procurement from a cadaveric source and also emphasizes the difficulties associated with the use of nonsibling donors.

MATERIALS AND METHODS

Case report. A 5-year-old boy with acute lymphocytic leukemia attained a remission with conventional therapy that continued for 3 years. Subsequently, he experienced three bone marrow relapses, all successfully treated with standard reinduction and maintenance therapies. His course was notable for methotrexate-associated hepatic cirrhosis, portal hypertension, colitis, hemorrhagic cystitis, and *Streptococcus viridans* septicemia with cardiac arrest. At age 12, upon recovery, he was transferred to the University of Minnesota Hospital for BMT.

No histocompatible siblings were available to serve as a bone marrow donor, but his father was phenotypically histocompatible for all class 1 (HLA-A, -B, -C,) and class 2 (HLA-DR, -DQ, -DP) antigens tested and was, therefore, selected as the donor. The donor and recipient were mutually nonreactive in MLC, with relative response rates of less than 5%. The donor was 40 years old, previously healthy, and had no risk factors for cardiac disease; however, while awaiting marrow harvesting, he suffered an acute myocardial infarction. Vigorous resuscitative efforts were unsuccessful in restoring cardiovascular pressure or respiration. No red cell transfusions were administered during resuscitation or harvesting. In accord with the father's last expressed desires, 900 mL of bone marrow was harvested 40 minutes after cessation of cardiopulmonary resuscitation. After transportation for two hours at room temperature, the marrow was processed, and bone marrow mononuclear cells were stored at −180 °C.[28] Aliquots were thawed and tested in cell culture on the day of marrow infusion. With the consent of the University of Minnesota Human Subjects Committee, the family was informed of the potential risks of graft rejection and partial engraftment because of the circumstances of the marrow procurement and requested that the BMT procedure proceed as

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originally planned. Pretransplant conditioning consisted of cytosine arabinoside (3 g/m^2/dose × 12) beginning on day −8, and 8.5 Gy single-dose total-body irradiation (TBI) delivered at 26 cGy/min on day −1. On the day of BMT, 2.96 × 10^8 cells/kg bone marrow were infused. Methotrexate, antithymocyte globulin, and prednisone were administered as GVHD prophylaxis.[29]

The initial month after BMT was complicated by gastrointestinal mucositis, ileus, bacterial sepsis, and persistent fevers. Normalization of peripheral blood counts occurred 26 days post-BMT (Fig 1). An absolute reticulocyte count of greater than 70,000 occurred on day 27 post-BMT. However, the patient was platelet transfusion dependent at this time and on all subsequent days post-BMT. A bone marrow biopsy specimen 28 days post-BMT was hypocellular with a decrease in erythroid cells, myeloid cells, and megakaryocytes. Concurrently with a sudden rise in the WBC count on day 37 post-BMT, profuse diarrhea and abdominal pain developed along with a reticular, morbilliform erythematous eruption over the trunk, extremities, and soles. Biopsy specimens of the skin, esophagus, stomach, and rectum were all consistent with acute GVHD. Immunoperoxidase stains and viral cultures were negative for viruses.

Treatment for GVHD was initiated with 2 mg/kg/d of methylprednisolone and escalated to 88 mg/kg/d before improvement of the skin lesions and partial resolution of diarrhea were noted. During this period, increased abdominal distress, hyperamylasemia, renal failure requiring dialysis, antibody-negative hemolytic anemia, disseminated intravascular coagulation, and pneumonia with progressive cardiorespiratory deterioration developed. Fiber-optic bronchoscopy was significant for single septate hyphae, and urinalysis was positive for *Aspergillus* ustus and a *Kloeckera* species. He died on the 67th day post-BMT of cardiorespiratory failure. Permission for an autopsy was denied.

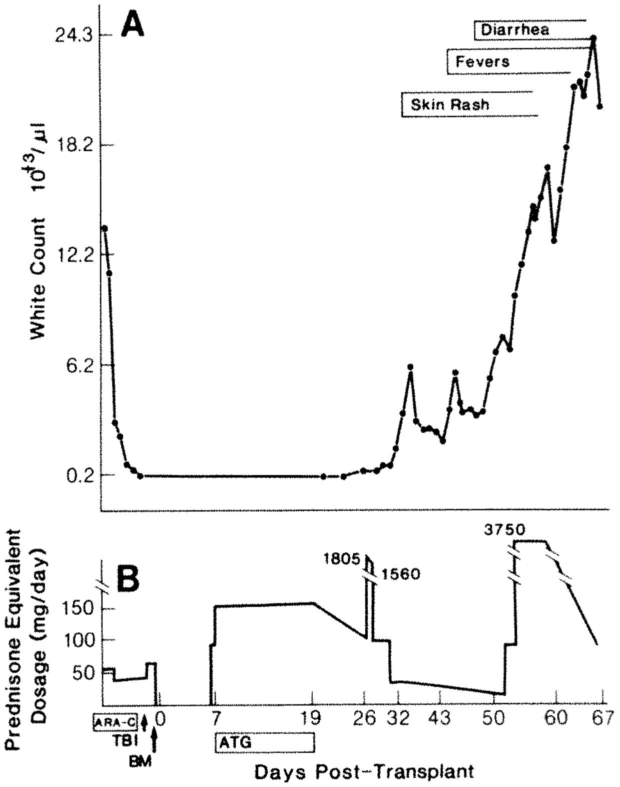

Fig 1. Peripheral leukocyte counts and recipient's GVHD course posttransplant. In (A), the peripheral blood leukocyte counts in relationship to the days posttransplant are depicted. Leukocyte counts of 1.0 × 10^{-3}/µL occurred 26 days posttransplant. The leukocyte count began to escalate with signs of GVHD. (B) presents the dose of steroids used for GVHD prophylaxis, day 6 to day 52, and treatment, day 53 until the day of death, 67 days posttransplant.

Typing for HLA-A, -B, -C, -DR, -DP, -DQ. Typing for class 1 (HLA-A, -B, -C) and class 2 (HLA-DR, -DP, -DQ) antigens was performed using a two-stage microcytotoxicity assay as previously described.[30,31] Class 1 antigen typing was performed on mononuclear cells, whereas class 2 antigen typing was accomplished using nylon wool–purified B lymphocytes.

Bone marrow processing. Bone marrow was aspirated from the posterior iliac crest and mixed with Hanks' balanced salt solution, 5% human serum albumin, and 100 U heparin/mL. The mixture was filtered and centrifuged at 4640 g for ten minutes. The buffy coat cells were resuspended in Hanks' balanced salt solution with 10% dimethylsulfoxide and 5% human serum albumin before cryopreservation. The methods for cryopreservation and thawing have been previously described.[28] No modifications of the marrow processing procedure were made. Bone marrow cryopreservation was started seven hours postmortem. Routine bacterial culture and marrow cell viability (as assessed by trypan blue exclusion) were performed.

Cell culture techniques. Techniques for bone marrow multipotent CFU-MIX (CFU-GEMM) and committed myeloid (CFU-GM) and erythroid (BFU-E and CFU-E) in vitro progenitor culture have been previously described and were done according to the method of Messner with modifications.[27,32-34] CFU-E assays were performed in a plasma clot assay as previously described.[35]

Engraftment studies. DNA genotypic analysis was accomplished post-BMT by identifying donor-specific restriction fragment length polymorphisms.[36,37] Peripheral blood mononuclear cells and granulocytes were isolated by differential gradient centrifugation (Ficoll-Hypaque; density, 1.077) as previously described.[37] Methods for the isolation of high-molecular weight DNA, restriction enzyme digestion, gel electrophoresis, capillary transfer of digested fragments onto nylon membranes, hybridization of the radiolabeled probes, and stringency washes of the filter membrane have been described in detail.[37]

Probes D9S1, D14S1, D15S1, D17S1, and D20S1 were generously provided by Dr Ray White (University of Utah and the Howard Hughes Institute). Probes D12S4 and D13S2 were kindly provided by Dr Webster Cavenee (University of Cincinnati). Description of these probes, alleles detected, and hybridization conditions have been previously reported.[38-40] Probes binding to informative alleles from Eco R1–generated polymorphisms are D12S4 and D14S1, whereas D13S2, D15S1, D17S1, and D20S1 are informative with Msp 1, and D9S1 and D13S2 are informative with Taq 1 digestion of genomic DNA.

Chromosomal analysis was performed on the donor and recipient pre-BMT using quinacrine-banding techniques.[41] No donor-recipient polymorphisms were identified, and therefore, no post-BMT studies were obtained.

RESULTS

A total of 1.94 × 10^{10} nucleated marrow cells were obtained 40 minutes postmortem. Lymphocytes comprised 11.3% of the original marrow specimen, monocytes 4.6%, neutrophil and neutrophil precursors comprised 62.3%, and erythroblasts were 15.4% of the total marrow cell population. Viability was noted to be greater than 99% by trypan blue dye exclusion. Buffy coat isolation resulted in a 27% loss of cells. At the time of the second thawing and infusion into the recipient, the marrow consisted of 81% mononuclear cells, 7% neutrophil and neutrophil precursors, and 9% normoblasts. Routine bacterial cultures were negative.

Fresh progenitor cell cultures all yielded values that were greater than or equal to median values for our laboratory. Preservation of CFU-MIX, BFU-E, CFU-E, and CFU-GM were present at levels of 15% to 20% of the fresh marrow sample at the time of BMT. The total number of progenitor cells in the infusion was 0.2, 3.7, 11.8, and 3.8×10^3/kg for CFU-MIX, BFU-E, CFU-E, and CFU-GM, respectively.

Engraftment studies. Pre-BMT, the donor and recipient were studied for polymorphic differences of red blood cell phenotypes and genotypic polymorphisms by cytogenetic techniques and by Southern blotting analysis. Red cell phenotyping revealed a difference in Rhesus blood typing of the donor (D−) and the recipient (D+). However, post-BMT specimens were not obtained, and therefore, red cell engraftment cannot be evaluated. Cytogenetic analysis with Q-banding techniques demonstrated no donor-recipient polymorphisms and, therefore, was not a useful marker for engraftment in this patient.

Southern blotting analysis of donor and recipient was performed using three different restriction endonucleases—Eco R1, Msp 1, and Taq 1. These were selected since there is a higher likelihood of informative alleles detectable with a variety of radiolabeled probes and initially noninformative blots could be reported with one or more radiolabeled probes. Screening of the donor and recipient identified a donor-specific polymorphism with D13S2 and D15S1. All other probes failed to identify unique donor or host fragments that could be used to monitor engraftment. Post-BMT, as illustrated in Fig 2, mononuclear cells and granulocytes were found to have the genotypic pattern of the donor.

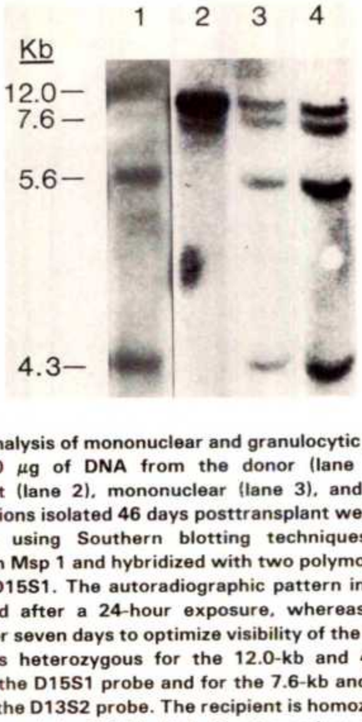

Fig 2. Analysis of mononuclear and granulocytic engraftment. Three to 10 μg of DNA from the donor (lane 1), recipient pretransplant (lane 2), mononuclear (lane 3), and granulocytic (lane 4) fractions isolated 46 days posttransplant were studied for engraftment using Southern blotting techniques. DNA was digested with Msp 1 and hybridized with two polymorphic probes, D13S2 and D15S1. The autoradiographic pattern in lanes 2 to 4 was obtained after a 24-hour exposure, whereas lane 1 was reexposed for seven days to optimize visibility of the DNA pattern. The donor is heterozygous for the 12.0-kb and 4.3-kb alleles detected by the D15S1 probe and for the 7.6-kb and 5.6-kb allele detected by the D13S2 probe. The recipient is homozygous for the 12.0-kb allele and the 7.6-kb allele. Posttransplant, mononuclear, and granulocyte fractions are consistent with a donor origin of these fractions, although up to 25% of residual host cells may be present and not detectable by this technique because of the lack of a host-specific polymorphism.

Despite the lack of host-specific fragments, it is likely that if there were residual host cells present post-BMT, these cells would constitute <25% of the overall population based on the relative intensity of the different fragments in the post-BMT samples. In this instance, the use of two probes on the blot provides an internal control for the assessment of engraftment based on the relative intensities of the different alleles (Fig 2). Since the host is homozygous for the alleles to which these probes bind, an even lower percentage of host cells should be detectable in this system. We conclude from these studies that the host was repopulated with greater than 75% donor mononuclear and granulocytic precursor cells, although partial engraftment at a level of up to 25% cannot be discounted. Further screening for host-specific polymorphisms was not possible because of the small amount of recipient pre-BMT DNA available. Lastly, the presence of biopsy-proven multi-organ system GVHD, particularly in the absence of viral infection, is further suggestive (but indirect) evidence that donor cells were present in substantial numbers.

DISCUSSION

Engraftment of cells from at least two lineages occurred in this recipient of paternal, cadaveric marrow. Cadaveric marrow as a feasible source of donor marrow has been infused into animal models in vitro and in vivo since the availability of cryopreservation techniques in the 1950s.[42,43] The first human cadaveric marrow infusion was performed by Thomas and colleagues in 1957.[44] Cadaveric marrow obtained three hours postmortem was frozen for 3 weeks, and 3.17×10^9 cells were infused throughout a 3-day period into a 59-year-old man with chronic lymphocytic leukemia who was conditioned with 4.5 Gy TBI divided throughout eight days. The patient had an apparent temporary erythrocyte engraftment of less than 1-month duration. Ferrebee and co-workers compared the cell yields and viability of marrow obtained from the human fetus, the usual rib removed at surgery, or ilium aspiration from adult cadaveric donors whose marrow cells were removed and frozen up to six hours postmortem.[45] Adequate numbers of viable cells were obtainable from each of these sources postmortem, especially if procured within four hours postmortem. The feasibility of marrow procurement at the time of cadaveric kidney harvesting was reported by Monaco and colleagues.[46] These investigators infused cadaveric marrow following kidney transplantation as a means of inducing donor-specific tolerance in the setting of zero-antigen matching. In their patient, donor erythrocytes persisted up to 2 months postreinfusion, although there was no evidence of lymphoid engraftment despite the use of antilymphocyte serum, prednisone, and azathioprine as preinfusion immunosuppression. As suggested by the case descriptions of Thomas, et al and Monaco and colleagues, erythroid engraftment is possible following cadaveric marrow infusion.

The pluripotent capacity of cadaveric marrow was not evident, however, in these preliminary studies. Liu et al documented the ability of murine cadaveric marrow obtained at varying times postmortem to produce day 7 (committed cell lineage) and day 10 (multilineage) splenic colonies in lethally irradiated mice.[47,48] Human cadaveric marrow[49] has been shown to be capable of generating erythroid and committed myeloid colonies up to 19 hours postmortem in colony assays (BFU-E and CFU-GM, respectively).

Leukocyte engraftment as denoted by a leukocyte count $>10^3/\mu L$ occurred prior to day 28 post-BMT, which is within the normal range of age- and disease-matched controls in our institution (Filipovich, unpublished observations). Engraftment of mononuclear cells and granulocytes was demonstrated in our patient by Southern blotting techniques. The extent of engraftment of these two lineages was comparable. In other patients, we have observed differences in the degree of engraftment of mononuclear cells and granulocytic cells in cases in which the recipient was at higher risk for incomplete engraftment or graft rejection based on the pre-BMT cytoreductive regimen used or the use of T cell depletion of donor marrow for GVHD prophylaxis.[37]

The availability and feasibility of using cadaveric marrow would eliminate the potential, albeit small, risk of marrow donation and hospitalization of volunteer unrelated donors. Several centers have already begun to store cadaveric marrow.[26,27] However, it should be emphasized that the use of cadaveric donors poses several difficulties, particularly on a national scale. The probability of matching for HLA antigens is low, approaching 1 in 10,000 to 30,000. The cadaveric donor pool size required to provide potential recipients with phenotypically compatible marrow can be estimated from the available experiences of living, volunteer, unrelated donor pools. Data from the Anthony Nolan Unrelated Donor Registry in England, which has had an accrual rate of 5,000 volunteer donors per year since 1975 and now contains over 50,000 names, is thought to be of sufficient size to provide HLA-A, -B, -D/DR–compatible, MLC nonreactive donors for approximately 30% of the patients for whom a search is requested.[50]

In addition, the current complications of BMT from nonsibling donors would not be diminished with the use of cadaveric marrow and, therefore, would present formidable obstacles to the routine usage of this type of donor marrow. Of the less than 100 patients who have received transplants from unrelated donors, the overall survival rate is approximately 20%.[51] The major causes of mortality have been GVHD, infection, and leukemic relapse in this group of patients, many of whom had advanced disease.[51] Insufficient numbers of patients have received HLA phenotypically identical unmanipulated marrow from unrelated donors to permit definitive conclusions with respect to the morbidity and mortality for patients lacking histocompatible sibling donors. However, recent results of the Seattle group in their experience with related donors who were not HLA-identical siblings are encouraging.[52] Donor and recipient pairs who were phenoidentical but genotypically nonidentical had a similar incidence of acute GVHD as compared with controls who received genotypically identical marrow. The ultimate success of this procedure may rely, in part, on the development of more effective methods of GVHD prophylaxis and treatment and, perhaps, more sophisticated HLA typing.[53] Whether these problems can be solved to permit the routine collection of cadaveric marrow by institutions in the near future is not certain.

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REFERENCES

1. Hansen JA, Clift RA, Beatty PG, Mickelson EM, Nisperos B, Martin PJ, Thomas ED: Marrow transplantation from donors other than HLA genotypically identical siblings, in Gale RP (ed): Advances in Bone Marrow Transplantation. New York, Liss, 1983, p 739

2. Filipovich AH, Ramsay NKC, McGlave PB, Quinones R, Winslow C, Heinitz KJ, Arthur D, Kersey JH: Mismatched bone marrow transplantation at the University of Minnesota: Use of related donors other than HLA/MLC identical siblings and T-cell depletion, in Gale RP (ed): Advances in bone marrow transplantation. New York, Liss, 1983, p 769

3. Dupont B, O'Reilly RJ, Pollack MS, Good RA: Use of HLA genotypically different donors in bone marrow transplantation. Transplant Proc 11:219, 1979

4. O'Reilly RJ, Kapoor N, Pollack M, Sorell M, Chaganti RSK, Blaese RM, Wank R, Good RA, Dupont B: Reconstitution of immunologic function in a patient with severe combined immunodifficiency following transplantation of marrow from an HLA-A, B, C nonidentical but MLC-compatible paternal donor. Transplant Proc 11:1934–1937, 1979

5. Filipovich AH, Vallera D, Youle R, Quinones R, Neville D, Kersey JH: Ex vivo T-cell depletion with immunotoxins in allogeneic bone marrow transplantation (BMT): The pilot clinical study for prevention of graft-versus-host disease. Transplant Proc 17:442, 1985

6. Fisher A, Durandy A, Villartay JP, Vilmer E, Gerota I, Griscelli C: HLA-haploidentical bone marrow transplantation for severe combined immunodeficiency using E-rosette fractionation and cyclosporin. Transplant Proc 447, 1985

7. Sondel PM, Bozdech MJ, Trigg ME, Hong R, Finlay JL, Kohler PC, Longo W, Hank JA, Billing R, Steeves R, Flynn B: Additional immunosuppression allows engraftment following HLA-matched T-cell depleted bone marrow transplantation for leukemia. Transplant Proc 17:460, 1985

8. O'Reilly RJ, Collins NH, Kernan N, Brochstein J, Dinsmore R, Kirkpatrick D, Siena S, Keever C, Jordan B, Shank B, Wolf L, Dupont B, Reisner Y: Transplanation of marrow-depleted T-cells by soybean lectin agglutination and E-rosette depletion: Major histocompatibility-related graft resistance in leukemic transplant recipients. Transplant Proc 17:455, 1985

9. Copenhagen Study Group of Immunodeficiencies: Bone-marrow transplantation from an HLA non-identical but mixed-lymphocyte-culture identical donor. Lancet 1:1146, 1973

10. Vossen JM, de Koning J, van Bekkum DW, Dicke KA,

Eysvoogel VP, Hijmans W, van Loghem E, Radl J, van Rood JJ, van der Waay D, Dooren LJ: Successful treatment of an infant with severe combined immunodeficiency by transplantation of bone marrow cells from an uncle. Clin Exp Immunol 13:9, 1973

11. Niethammer D, Goldmann SF, Haas RJ, Dietrich M, Flad H-D, Fliedner TM, Kleihauer E: Bone marrow transplantation for severe combined immunodeficiency with the HLA A–incompatible but MLC-identical mother as a donor. Transplant Proc 8:623, 1976

12. Geha RS, Malakian A, LeFranc G, Chayban D, Serre JL: Immunologic reconstitution in severe combined immunodeficiency following transplantation with parental bone marrow. Pediatrics 58:451, 1976

13. Powles RL, Morgenstern GR, Kay HEM, McElwain TJ, Clink HM, Dady PJ, Barrett A, Jameson B, Depledge MH, Watson JG, Sloane J, Leigh M, Lumley H, Hedley D, Lawler SD, Filshie J: Mismatched family donors for bone marrow transplantation as treatment for acute leukemia. Lancet 1:612, 1983

14. Storch S, Gale RP, Champlin R, Ho W, Falk PM, Feig SA: Partially mismatched marrow transplantation for acute leukemia and aplastic anemia, in Gale RP (ed): Advances in Bone Marrow Transplantation. New York, Liss, 1983, p 785

15. Speck B, Swaan FE, van Rood JJ, Eernisse JG: Allogeneic bone marrow transplantation in a patient with aplastic anemia using a phenotypically HLA-identical unrelated donor. Transplantation 16:24, 1973

16. Lohrmann HP, Dietrich M, Goldmann SF, Kristensen T, Fliedner TM, Abt C, Pflieger H, Flad H-D, Kubanek B, Heimpel H: Bone marrow transplantation for aplastic anaemia from an HLA and MLC-identical unrelated donor. Blut 31:347, 1975

17. Horowitz SD, Groshong T, Bach FH, Hong R: Treatment of severe combined immunodeficiency with bone marrow from an unrelated, mixed-leukocyte-culture non-reactive donor. Lancet 2:431, 1975

18. O'Reilly RJ, Dupont B, Pahwa S, Grimes E, Smithwick EM, Pahwa R, Schwartz S, Hansen JA, Siegal FP, Sorell M, Svejgaard A, Jersild C, Thomsen M, Platz P, L'Esperance P, Good RA: Reconstitution in severe combined immunodeficiency by transplantation of marrow from an unrelated donor. N Engl J Med 297:1311, 1977

19. Hansen JA, Clift RA, Thomas ED, Buckner CD, Storb R, Giblett ER: Transplantation of marrow from an unrelated donor to a patient with acute leukemia. N Engl J Med 303:565, 1980

20. Gordon-Smith ED, Fairhead SM, Chipping PM, Hows J, James DC, Dodi A, Batchelor JR: Bone marrow transplantation for severe aplastic anaemia using histocompatible unrelated volunteer donors. Br J Med 185:835, 1982

21. Duquesnoy RJ, Zeevi A, Marrari M, Hackbarth S, Camitta B: Bone marrow transplantation for severe aplastic anemia using histocompatible unrelated volunteer donors. Transplantation 35:566, 1983

22. Gingrich RD, Howe CWS, Goeken NE, Ginder GD, Kugler JW, Tewfik HH, Klassen LW, Armitage JO, Fyfe MA: The use of partially matched, unrelated donors in clinical bone marrow transplantation. Transplantation 39:526, 1985

23. McCullough J. Bone marrow transplantation from unrelated volunteer donors: Summary of a conference of scientific, ethical, legal, financial, and other practical issues. Transfusion 22:78, 1982

24. Bortin MM, Buckner CD: Major complications of marrow harvesting for transplantation. Exp Hematol 11:916, 1983

25. Buckner CD, Clift RA, Sanders JE, Stewart P, Bensinger WI, Doney KC, Sullivan KM, Witherspoon RP, Deeg HJ, Appelbaum FR, Storb R, Thomas ED: Marrow harvesting from normal donors. Blood 64:630, 1984

26. Maples JC, Reynolds CP, Matthews JG, Lewis SB, Woody JN: High dose chemotherapy and autologous bone marrow transplantation workshop. Transplantation Research Program Center, Naval Medical Research Institute, Bethesda, Md, 1984

27. Mugishima H, Terasaki P, Sueyoshi A: Bone marrow from cadaver donors for transplantation. Blood 65:392, 1985

28. Lasky LC, Ash RC, Kersey JH, Zanjani ED, McCullough J: Collection of pluripotent hematopoietic stem cells by cytapheresis. Blood 59:822, 1982

29. Ramsay NCK, Kersey JH, Robison LL, McGlave PB, Woods WC, Krivit W, Kim TH, Goldman AI, Nesbit ME: A randomized study demonstrating the influence of treatment regimen and age. N Engl J Med 306:392, 1982

30. Bodmer JG, Rickbourne P, Richards S: Joint report on Ia serology, in Bodmer WR (ed): Histocompatibility Testing. Copenhagen, Munksgaard, 1977

31. O'Leary J, Reinsmoen NL, Yunis EJ: Mixed lymphocyte culture reaction, in Rose NP (ed): Manual of Clinical Immunology. Washington DC, American Society of Microbiology, 1976, p 820

32. Messner HA, Fauser AA: Culture studies of human pluripotent hematopoietic progenitors. Blut 41:327, 1980

33. Fausner AA, Messner HA: Identification of megakaryocytes, macrophages, and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood 53:1023, 1979

34. Ash RC, Detrick RA, Zanjani ED: Studies of human pluripotential stem cells (CFU-GEMM) in vitro. Blood 58:309, 1981

35. Zanjani ED, Lutton JD, Hoffman R, Wasserman LR: Erythroid colony formation by polycythemia vera bone marrow in vitro. J Clin Invest 59:841, 1977

36. Ginsburg D, Antin JH, Smith BR, Orkin SH, Rappeport JM: Origin of cell populations following bone marrow transplantation: Analysis using DNA sequence polymorphisms. J Clin Invest 75:596, 1985

37. Blazar BR, Orr HT, Arthur DC, Kersey JH, Filipovich AH: Restriction fragment length polymorphisms (RFLPs) as markers of engraftment in allogeneic marrow transplantation. Blood 66:1436, 1985

38. Skolnick MH, Willard HF, Menlove LA: Report of the committee on human gene mapping by recombinant DNA techniques. Cytogenet Cell Genet 37:210, 1984

39. Barker D, Schager M, White R: Restriction sites containing CpG show a higher frequency of polymorphism in human DNA. Cell 36:131, 1984

40. Cavenee W, Leach R, Mohandas T, Pearson P, White R: Isolation and regional localization of DNA segments revealing polymorphic loci from human chromosome 13. Am J Hum Genet 36:10, 1984

41. Hozier JC, Lindquist LL: Banded karyotypes from bone marrow: A clinical useful approach. Hum Genet 53:205, 1980

42. Barnes DWH, Loutit JF: The radiation recovery factor: Preservation by the Polge-Smith-Parkes technique. JNCI 15:901, 1955

43. Ferrebee JW, Billen D, Urso IM, Lu WC, Thomas ED, Congdon CC: Preservation of radiation recovery factor in frozen marrow. Blood 12:1096, 1957

44. Thomas ED, Lochte HL, Lu WC, Ferrebee JW: Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 257:491, 1957

45. Ferrebee JW, Atkins L, Lochte HL, MacFarland RB, Jones AR, Dammin GJ, Thomas ED: The collection, storage, and preparation of viable cadaver marrow for intravenous use. Blood 14:140, 1959

46. Monaco AP, Clark AW, Wood ML, Codish SD, Brown RW: Possible active enhancement of a human cadaver renal allograft with antilymphocyte serum (ALS) and donor bone marrow: Case report of an initial attempt. Surgery 79:384, 1976

47. Liu PL, Ogawa M, Crook L, Ochia R, Upshur JK: Proliferative function of cadaveric bone marrow cells. Am J Hematol 5:145, 1978

48. Liu PL, Poon KC, Crook L, Hong CC, Eguchi M: Pluripotent hematopoietic murine postmortem bone marrow. Ann Clin Lab Sci 10:40, 1980

49. Yesus YW, Kneally C, Taylor HM: Preservation of hematopoetic stem cells in cadaveric marrow. Am J Clin Pathol 76:205, 1981

50. James DCO: Organization of a hospital bone marrow panel, in Sibinga CS, Das PC, Opelz G (eds): Transplantation and Blood Transfusion. Boston, Martinus Nijhoff, 1984, p 131

51. Mickey MR: Donor pool size and bone marrow transplantation, in Donor Registries for Bone Marrow Transplantation Program and Abstract Book. National Institutes of Health Technology Assessment Meeting, May 13, 1985, p 54

52. Beatty PG, Clift RA, Mickelson EM, Nisperos BB, Flournoy N, Martin PJ, Sanders JE, Stewart P, Buckner CD, Storb R, Thomas ED, Hansen JA: Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 313:765, 1985

53. Cairns JS, Curtsinger JM, Dahl CA, Alter BJ, Bach FH: Sequence polymorphism of HLA DRb1 alleles relating to T-cell-recognized determinants. Nature 317:166, 1985

Double Inheritance of an Alpha I/65 Spectrin Variant in a Child With Homozygous Elliptocytosis

By Michel Garbarz, Marie Christine Lecomte, Didier Dhermy, Claude Feo, Isabelle Chaveroche, Huguette Gautero, Odile Bournier, Christiane Picat, Anne Goepp, and Pierre Boivin

Hemolytic anemia with red cell fragmentation, poikilocytosis, and elliptocytosis was discovered in a 6-week-old black infant. Both parents and a brother of the propositus had compensated mild Hereditary Elliptocytosis (HE). Elliptocytosis was prominent in the proband's father with the presence of numerous rod-shaped cells whereas, in the proband's mother, elliptocytosis was less marked and cells were less elongated than in the father. The proband's red cells fragmented at 45 °C instead of 49 °C for control cells. Both the parents' and brother's red cells fragmented at 47 °C. The deformability of the proband's red cells was markedly reduced when measured with the ektacytometer; the red cells of both the proband's parent and brother exhibited an intermediate decrease in red cell deformability. Spectrin self-association was defective in the propositus as well as in his parents and brother. Limited tryptic digestion of the proband's spectrin, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), revealed a complete absence of the normal 80,000 dalton alpha I domain and the presence of an abnormal 65,000 dalton peptide. Two-dimensional isoelectric focusing/SDS-PAGE of limited tryptic digests of spectrin from both the proband's parents and brother revealed a decrease in the normal 80,000 alpha I domain and the presence of the 65,000 peptide variant. On the basis of biochemic studies performed on the patients' spectrin, we concluded that the proband had homozygous HE, having inherited the structural defect of spectrin present in a heterozygous state in each of his parents. On a clinical and morphologic level, homozygous HE imitates two other forms of congenital hemolytic anemia associated with a spectrin self-association defect: HE with pycnocytosis in infancy and Hereditary Pyropoikilocytosis. This report emphasizes the importance of confronting clinical and rheological as well as biochemical investigations in studying and discussing different entities.

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IT IS now known that normal red cell stability and deformability depend upon a submembranous network of proteins called the skeleton, which is attached to the inner surface of the red cell membrane.[1] Spectrin, the predominant protein of the skeleton, forms long fibers that are mainly spectrin tetramers formed by two heterodimers joined "head to head." Each dimer is composed of two different chains denoted α and β. The distal ends of spectrin are linked together by short polymers of actin. Protein band 4.1 binds to the distal end of the spectrin molecule and regulates the interactions between spectrin and actin. The spectrin-actin-protein 4.1 network is attached to the membrane via ankyrin, a protein that binds spectrin to the anion channel transmembrane protein band 3.

The effect of the skeleton on cell stability has been illustrated by recently discovered hereditary deficiency, or dysfunction, of membrane skeletal proteins in patients with congenital hemolytic anemia[2] such as Hereditary Elliptocytosis (HE),[3-13] Hereditary Pyropoikilocytosis (HPP),[14-19] and Hereditary Spherocytosis (HS).[20-22] The most frequent type of Hereditary Elliptocytosis, denoted mild HE, is an autosomal dominant disorder characterized by prominent elliptocytosis and absent-to-moderate hemolysis.[2,13,23] Some patients with HE may have overt hemolysis and anemia, which are related to the red cell fragmentation. This hematologic picture can be observed in distinct clinical entities such as mild HE with poikilocytosis in infancy, homozygous HE, or HPP, a disorder closely related to HE.[2,13,23]

A few patients with homozygous HE have been reported.[24-29] The patients had severe hemolytic anemia with marked red cell fragmentation, poikilocytosis, and elliptocytosis, and both parents of the patients had classical mild HE. In three families, the skeletal protein defect was determined. Féo and Tchernia[3,4] reported three young patients in whom homozygous HE was associated with a complete absence of protein band 4.1. The parents had mild HE and exhibited a partial deficiency in protein band 4.1. Evans and colleagues[28] reported three children with homozygous HE and profound defective spectrin dimer-dimer association. The parents had classical mild HE and displayed a lesser but significant defect in spectrin self-association (the cases of heterozygous HE associated with defective spectrin self-association have been termed type I HE.)[8] Dhermy and colleagues[29] reported a patient with severe transfusion-dependent poikilocytic hemolytic anemia. The parents had mild HE and exhibited defective spectrin self-association; this defect was associated in both parents with a structural alteration in the spectrin molecule: abnormal patterns in tryptic digestion of spectrin were observed, namely, a decrease in the amount of the 80 Kd peptide of the spectrin α chain involved in dimer-dimer association and a corresponding increase in a 74 Kd peptide derived from the same region.

We have now investigated another patient with homozygous type I HE. This patient exhibited defective spectrin self-association, and peptide maps of his spectrin tryptic digests revealed a complete absence of the normal 80 Kd peptide, and the presence of an unusual peptide of 65 Kd. The patient's father and mother had typical mild HE and displayed defective spectrin self-association, associated with

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a decrease in the normal 80 Kd α I domain and the presence of the 65 Kd variant of spectrin α chain. We discuss this case with the aid of clinical, rheological, and biochemical investigations and in the light of two other kinds of congenital poikilocytic anemia with a spectrin self-association defect, HE with pycnocytosis in infancy and HPP.

CASE REPORT

This family is of Malian extraction. The propositus Mar.S., a female baby, was born in August 1984. Delivery was normal, and no congenital defect was apparent. At day six, a moderate icterus was noted. Hemoglobin was 12.5 g/dL, packed cell volume (PCV) 35%, and mean cell volume (MCV) 90 fl. Five weeks later, she was referred to the hospital for anemia. Clinical examination was found to be normal. The spleen was not palpable. Hb was 8.4 g per dL, red blood cell count was 2.9×10^{12}/L, PCV was 23.4% and MCV 80 fl. The reticulocyte count was 2.5×10^{11}/L. Elliptocytes and poikilocytes were observed on blood smears.

The parents (mother Ka.S, father Dj.S.) were not related. They had no significant medical history. In both parents hematologic data were normal (Table 1). Elliptocytosis was prominent in the father, whereas in the mother, only few elliptocytes were present on blood smears. A brother of the propositus (A S) was 18 months old at the time of this study and had no medical history. Elliptocytosis was discovered fortuitously during a routine blood examination. Hb was 10.3 g/dL, with red blood cell count 4.5×10^{12}/L, MCV 65 fl, and reticulocytes 4.5×10^{10}/L. Serum iron concentration was 9 mmol/L (normal range 13 to 31 mmol/L). Hemoglobin electrophoresis was normal.

MATERIALS AND METHODS

Materials. Beta mercaptoethanol (BME), glutaraldehyde, ethylene diamine tetraacetic acid (EDTA), TRIS, glycerol sucrose for density gradient studies, and L-1 tosylamido phenylethyl chloromethyl ketone (TPCK) Trypsin (3.5 units per mg) were from Merck (Darmstadt, GFR); phenylmethylsulfonyl fluoride (PMSF) and Diisopropyl fluorophosphate (DFP) were from Sigma, St Louis. All materials used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Biorad Laboratories (Richmond, Calif). Ampholines were obtained from LKB (LKB-Bromma, Sweden). Patient samples of venous blood obtained were drawn in sterile tubes, anticoagulated with heparin, and used within 24 hours.

Methods. Routine hematologic determinations were obtained with a coulter counter model S. Reticulocyte counts were made after new methylene blue staining. Morphologic studies: erythrocytes were examined on dried smears stained with May Grunwald Giemsa and on wet smears as previously described.[29] Red cell thermal sensitivity was examined as described.[30] Deformability measurements (osmotic gradient ektacytometry): whole-cell deformability was measured in the ektacytometer[31,32] as a continuous function of the suspending medium osmolality as previously described.[33]

Preparation of the erythrocyte membranes: the erythrocytes were washed three times in 5 mmol/L $NaPO_4$, 147 mmol/L NaCl pH 8.0. The ghosts were prepared according to Litman[34] except that 0.3 mmol/L PMSF was added to the lysis buffer.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of isolated membranes was performed using a 5% to 15% polyacrylamide gradient slab gel according to Laemmli.[35] Gels were stained by Coomassie blue.[36] To estimate spectrin/band 3 ratios, gels were quantified on a DU8 Beckman (Gagny, France) integrating densitometer.

Spectrin interaction studies. Spectrin was extracted by incubating the membranes in low ionic strength buffer either at 37 °C or at 4 °C.[15] Spectrin dimer-dimer association in solution was studied directly in the 37 °C extracts. The samples were incubated for 240 minutes at 30 °C to induce spectrin dimer-to-tetramer transformation. Dimer and tetramer species were separated by sucrose gradient velocity centrifugation and equilibrium constant Ka was determined as described.[29] The distribution of spectrin species in the 4 °C extracts was studied using nondenaturant gel electrophoresis (NDGE) and the percentage of spectrin dimer was determined as described.[29]

Limited tryptic digestion of spectrin. Crude spectrin extracts (1 mg/mL) were digested at 0 °C for 20 h in 20 mmol/L $NaPO_4$, 100 mmol/L NaCl pH 8.0. The enzyme/substrate ratio was 1/100 (w/w). The digestion was ended by adding of DFP (1 mmol/L final concentration). Each assay was done in duplicate. Spectrin peptides from the tryptic digestion were separated on a 7% to 15% polyacrylamide gradient gel as described.[29] Gels were stained with Coomassie brillant blue and the bands were quantified on a DU8 Beckman (Gagny, France) integrating spectrophotometer.

Isoelectric focusing. Isoelectric focusing followed by SDS-PAGE was performed according to O'Farrell's method[37] with the modifications reported by Speicher.[38] Samples of tryptic digest were lyophilized and solubilized in 9.5 mol/L urea, 2% Triton X 100, 5% BME, and 2.4% ampholines, pH 3.5–10, 4–6, 5–7, respectively. Samples were electrofocused for 16 hours at 340 volts in 4% polyacrylamide tube gels (0.5×13 cm) containing 2.4% ampholytes. The second dimension was performed on 7% to 15% linear gradient slab gels ($140 \times 100 \times 1.5$ mm) as described.[29] Gels were stained with Coomassie blue.[36]

RESULTS

Hematologic indices of the four members of the family were obtained at the time of the biochemic investigations. The results are summarized in Table 1.

Morphologic studies. On wet preparations, the morphology of the proband's red cell was characterized by the presence of numerous elliptocytes of different sizes and of various degrees of ellipticity, triangle shaped cells, and microcytes (Fig 1). The calculated MCV was 77.5 fl. But the distribution curve was bimodal, showing a proportion of about 20% of very microcytic red cells. This percentage corresponded with the number of microcytes observed on the wet preparation. The dispersion of the volumes is very broad since the proband's curve is superimposed on that of the father in the area where the volumes exceed the normal mode (>90 fl.; Fig 2). In the father, elliptocytosis was prominent (about 100%) with cells moderately to extremely elongated; no fragmented cells were observed. In the mother, elliptocytosis was less important (about 30%) with a majority of roundish cells; 3% to 5% of the cells were rod-shaped. The proband's brother's red cells were mainly heterogeneous

Table 1. Hematologic Investigations

	Hb (g/dL)	Red Cell Count ($\times 10^{2}$/L)	Mean Cell Volume (fl)	Reticulocytes (n/L)
Father (DS)	14.4	4.76	93	47,600
Mother (KS)	12.5	4.42	83	75,000
Proband (MS)	8.4	2.90	80	252,000
Brother (AS)	10.3	4.50	65	45,000

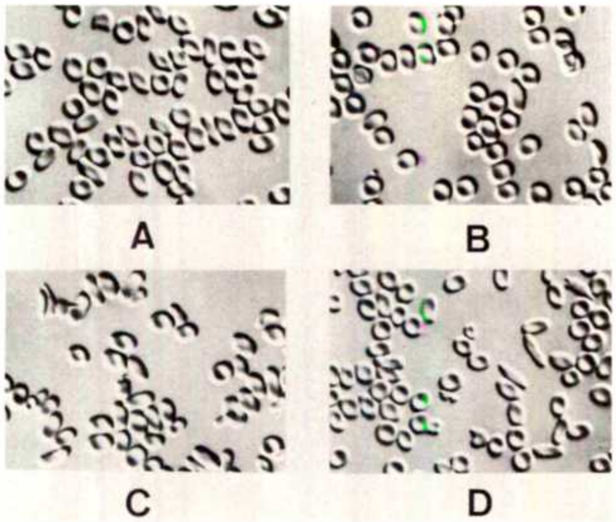

Fig 1. Morphology of erythrocytes from the four family members. Father (A) Mother (B) Proband (C) Proband's brother (D).

elliptocytes (about 70%) which were moderately elongated; few microcytes and schizocytes were present.

Red cell thermal sensitivity. Red cell thermal sensitivity was abnormal in the four patients. The most fragmentable cells were found in the proband: her red cells began to fragment at 45 °C (instead of 49 °C for control red cells). In both the proband's parents and brother, the critical temperature of red cell fragmentation was 47 °C.

Red cell deformability studies. The deformability of the red cells is expressed as an ektacytometric index (EI). When EI is plotted versus the osmolality of the suspending medium, the curve obtained shows three remarkable points (Fig 3): the EI in isotonicity (ISO), the minimum index in hypotonicity (HYPO) around 140 mOsm kg^{-1}, which corresponds to the maximum volume of the near-spherical cells just prior to hemolysis, and the index in hypertonicity (HYPER), which corresponds to the osmolality at which EI equals half the normal maximum on the hypertonic arm of the curve. Red cell deformability was found to be abnormal in all four patients (Fig 3): the proband's father and mother displayed a decrease in red cell deformability with an EI ISO of 0.28 and 0.34, respectively (controls: 0.55 ± 0.04); EI improved steadily when the cells were under moderate hypotonic conditions but the optimum EI was shifted markedly toward hypotonic values (200 mOsm) and was always lower than that of the control. The two curves had an asymmetric trapezoid shape. In the proband cell deformability was markedly reduced with an EI of 0.15 and the ektacytometric profile was reminiscent of that of both parents with the same trapezoid shape. In these three patients, EI HYPO values were obtained at the same osmolalities as in controls (141 ± 5 mOsm kg^{-1}). The proband's microcytic brother exhibited an osmotic deformability profile similar to that of his parents (EI ISO at 0.28) but the EI HYPO was shifted to the left (120 mOsm kg^{-1}) (not shown). It is known that in iron-deficient cells, the EI HYPO value is obtained at a lower osmolality than that in normal cells.[39]

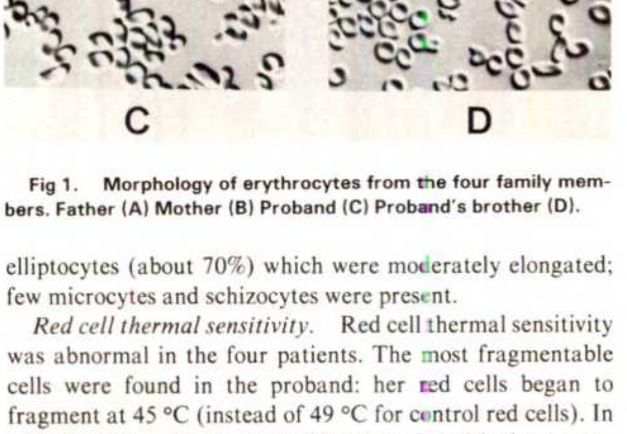

Fig 2. Red cell volume distribution measured by a Coulter C 1000 model E (Margency, France). The mean value was determined by integrating the area of the curve. We estimated the percentage of microcytes by comparing the area of the curve from the origin to channel 22 with the total area. Channel 22 (45 fl) corresponds to the channel under which less than 3% of normal cells are found. (C) curve of control. (M) Proband's mother's curve; (P) Proband's curve.

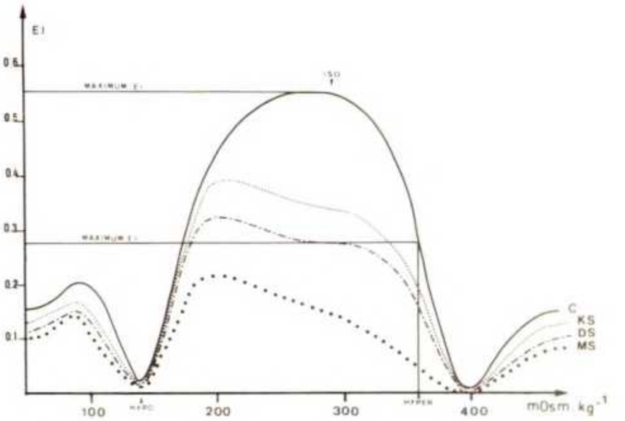

Fig 3. Changes in erythrocyte deformability as measured by the ektacytometric index (EI) during continuous variation of the suspending medium osmolality.[31] Curves of father (DS), mother (KS), proband (MS), are compared to control (C).

SDS-PAGE of the red cell membranes gave normal patterns in both the proband's parents and brother. The electrophoretic pattern of the proband showed a decrease in subcomponent 4.1a and an increase in subcomponent 4.1b related to the hypereticulocytosis. No reduction in the amount of spectrin relative to band 3 was observed in the proband or in either of her parents. Spectrin to band 3 ratio was 1.29 in the proband, 1.31 in the proband's father, and 1.37 in the proband's mother (spectrin to band 3 ratio in controls: 1.25 ± 0.22; n = 12).

Spectrin dimer-dimer association was defective in the four members of the family; the proportion of spectrin dimer was increased in the 4 °C extracts of the proband (41%) and in a lesser extent in both parents and brother (respectively, 28%, 28%, and 24% for a control value of 13% ± 3). The study of spectrin dimer self-association in solution performed only in the proband's mother and brother revealed a decreased association constant Ka, with a value of 3 and 1.8 × 10^5 mol/L^{-1}, respectively. (Control value: 6 ± 0.4 × 10^5 mol/L^{-1}). All results are summarized in Table 2.

Limited tryptic digestion of patient's spectrin. Limited tryptic digestion of crude spectrin extract from the proband revealed a complete absence of the 80 Kd peptide and the presence of an abnormal peptide of 65 Kd (Fig 4, right panel). In the three other members of the family (father, mother, and brother AS) we observed a decrease in the 80 Kd peptide and the presence of the 65 Kd peptide (Fig 4, left and right panels). Densitometric tracings (not shown) enabled us to estimate that the ratio 65/80 + 65 was 50% in the father, 40% (n = 4) in the mother, and 48% (n = 2) in the brother. Variations at the level of 35 to 37 Kd (decrease in the 35 and presence of a 37 Kd peptide) peptides were observed in the mother.

Two-dimensional electrophoresis (isoelectric focusing followed by SDS-PAGE) of tryptic digest of spectrin could only be performed in the parents and proband's brother. They confirmed the decrease in the 80 Kd peptide and the presence of the 65 Kd peptide which focused between pH 5.15 and 5.2 (Fig 5).

In the proband's mother we also observed variations in peptides related to the αII domain: a decrease of the two spots at 48 Kd level and the spot at 35 Kd, with the concomitant presence of two spots at 50 Kd and a spot at 37 Kd. The two spots at 50 Kd were also modified in their isoelectric point which was less acidic (Fig 5). These modifications corresponded to the "1/2" variant described by Knowles.[40]

DISCUSSION

We report the case of a child suffering from a congenital hemolytic anemia with poikilocytosis and elliptocytosis. The mean cell volume was slightly decreased and this corresponded with the presence of moderate fragmentation (Fig 2). This was associated with a normal EI HYPO. (Fig 3). The proband's erythrocytes were particularly sensitive to heat treatment (45 °C instead of 49 °C for normal erythrocytes). Both the proband's parents and brother exhibited mild HE and their red cells fragmented at 47 °C.

The diagnosis of homozygous HE was established in the proband with the aid of the biochemic studies performed in each member of the family. Both the proband's parents had defective spectrin self-association with an increased amount of spectrin dimer in the spectrin 4 °C extract. Peptide mapping of the tryptic digests of their spectrin showed a reduction of about 50% in the normal 80 Kd alpha I spectrin domain, and the presence of an abnormal 65 Kd variant. The proband exhibited defective spectrin self-association and had greater amounts of spectrin dimer in the spectrin 4 °C extracts than his heterozygous parents. The proband had inherited the structural alteration of spectrin from each of his parents, and the 80 Kd peptide was absent on the peptide maps of tryptic digests of his spectrin, only the 65 Kd variant being present. The proband's brother had mild HE and was heterozygous for the structural alteration of spectrin like each of his parents.

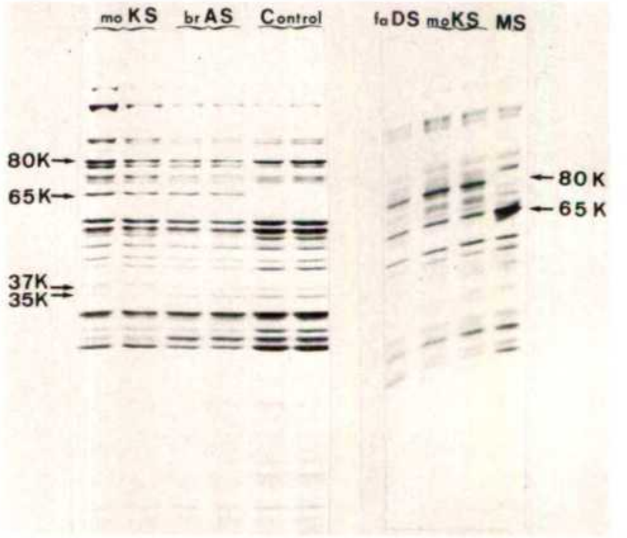

Fig 4. SDS-PAGE of limited tryptic digests of spectrin from proband (MS), proband's father (DS), proband's mother (KS), proband's brother (AS). Spectrin crude extracts were treated with TPCK trypsin (enzyme/substrate ratio: 1/100) at 0 °C for 20 hours. Gels were stained with Coomassie blue. The position of variable bands is indicated by arrows.

Table 2. Functional Studies of Spectrin

	Equilibrium Constants of Spectrin Dimer-Dimer Association in Solution (Ka 10^5 mol/L)	Percentage Spectrin Dimer in 4 °C Crude Spectrin Extract
Normal	6.00 ± 0.4 (n = 42)	13% ± 3% (n = 27)
Father (DS)	ND	28%
Mother (KS)	2.3	28% (n = 2)
Proband (MS)	ND	41%
Brother (AS)	1.8	24%

*ND, not determined.

This is the first reported case of homozygous type I HE with the spectrin alpha I/65 variant. This structural defect has been previously reported in seven cases of heterozygous HE,[41] and reports on three other HE patients with the same 65 Kd variant have been recently published.[42] The abnormal 65 Kd peptide was shown to derive from the 80 Kd alpha I domain.[41,42]

In the present case of homozygous HE, it was possible to follow the transmission of the spectrin structural defect from each heterozygous parent to the homozygous proband. In the cases of homozygous HE reported by Evans,[28] the probands were found to have twice the amount of spectrin dimer as their heterozygous parents but the type of the variant was not known. In the case of homozygous alpha I/74 reported by Dhermy,[29] the transmission of the defect could not be traced in the proband because the severity of the anemia required monthly transfusions. Interestingly, in this latter case and in Evans' cases (in which the type of the variant was not known), the proportion of spectrin dimer in the heterozygous parent was about 40%. In our present case, the proband's heterozygous alpha I/65 parents exhibited lower amounts of spectrin dimer and the homozygous proband had almost twice the spectrin dimer than did either of his parents. For

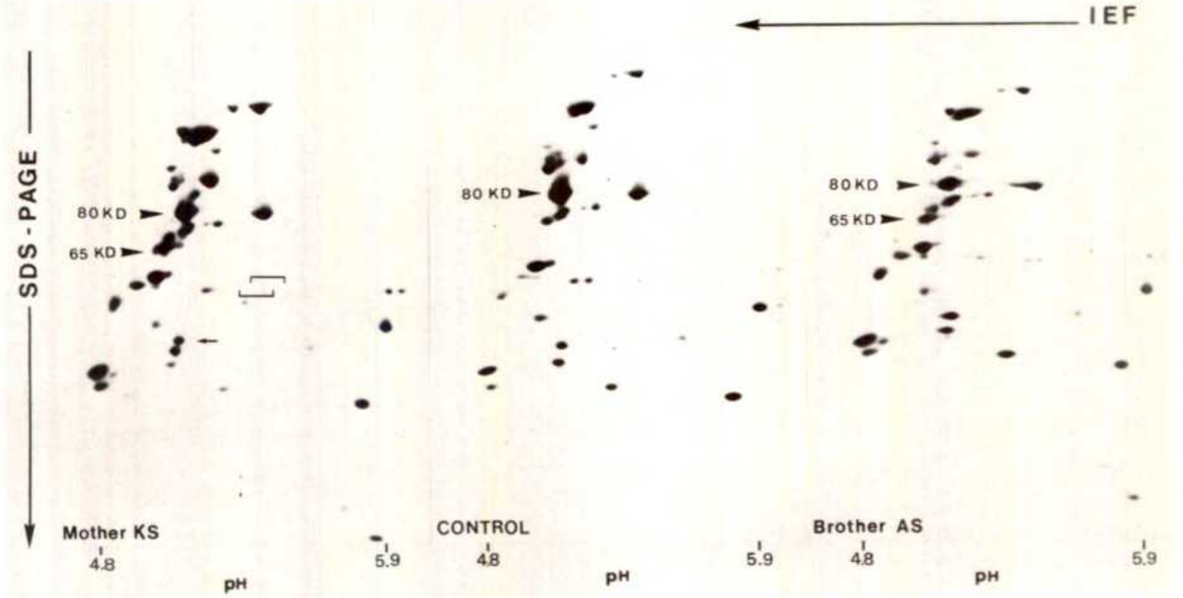

Fig 5. Two-dimensional isoelectric focusing/SDS-PAGE of tryptic digests (see Methods) of spectrin from proband's mother, from a control, and from proband's brother. The positions of the normal 80 Kd (α I) and the abnormal 65 Kd are indicated by arrowheads. On the ID IEF/SDS-PAGE of the proband's mother, heterozygous Type 2 polymorphism in the α II domain is shown between brackets (α II-T48/α II-T50). The tiny arrow indicates the subdomain α II-T37 just above the subdomain α II-T35.

technical reasons, we could not measure the spectrin dimer association constant in the homozygous proband, which would have been more meaningful in evaluating the functional defect in the alpha I/65 spectrin variant.

Two other forms of congenital hemolytic anemia associated with a spectrin self-association defect imitate the hematologic picture observed in homozygous HE: HE with pycnocytosis in infancy and HPP. In these two diseases, red cell fragmentation occurs at 43 °C to 45 °C. In HE with poikilocytosis in infancy, one parent has mild HE and exhibits the same increase in the amount of spectrin dimer and the same spectrin tryptic digest pattern as his poikilocytic offspring; after 12 to 14 months the morphologic poikilocytic picture evolves into a morphologic picture similar to that observed in the mild HE parent.[23,43] Structural studies on spectrin in HPP[16,18,19] have shown that one parent contains a structural defect in spectrin (74, 46, or 50 Kd variant) that is similar to a lesser extent to the HPP proband's defect. The other parent does not carry any identifiable defect but probably transmits to his HPP offspring an anomaly that acts in concert with the inherited membrane skeletal defect, resulting in the HPP phenotype. In the cases of HPP reported by Palek, spectrin self-association is more defective and the normal 80 Kd peptide is more reduced in the HPP proband than in the parent who carries the spectrin defect[16,18]; however, as we observed recently (unpublished data), the proportion of normal 80 Kd present may be the same in the HPP patient as in his parent or, as reported by Marchesi,[19] the 80 kD peptide can be totally absent in the HPP proband. In our case of homozygous HE, both the presence of 50% of the normal amount of 80 Kd peptide in each parent and the total absence of 80 Kd peptide in the proband are necessary to make the diagnosis of homozygous HE certain. Also, the amount of spectrin present in the proband's red cell membranes was normal, whereas this amount was found to be decreased (about 30%) in the HPP patients' red cell membranes.[13] In addition to the biochemic studies performed on each member of the family, we think that red cell deformability studies performed with the ektacytometer are very useful to confirm the diagnosis of homozygous HE in the proband: red cell deformability was more reduced in the proband than in her heterozygous parents (Fig 3)[4]; the trapezoid shape of the proband's red cell ektacytometric profile was reminiscent of the curve observed in each heterozygous HE parent. In our experience, this kind of asymmetric profile has so far been observed only in HE patients.[43] In our studies concerning HPP patients,[17] as well as in the recent case reported by Mentzer,[44] the ektacytometric profile observed in HPP is different from that of homozygous HE: the ektacytometric index in isotonicity is extremely reduced and the minimal ektacytometric index in hypotonicity is shifted to higher osmolality values (this being consistent with the increased osmotic fragility of red cells in HPP).

In the present case of homozygous HE, biochemical studies performed on patients' spectrin have shown that the homozygous proband had inherited the structural defect of spectrin present in a heterozygous state in each of his parents.

One interesting question to be asked would be whether the kind of spectrin molecular variant plays a role in the clinical severity of the disease. It has to be pointed out that in the case reported by Dhermy,[29] the proband had severe transfusion-dependent hemolytic anemia and was homozygous for a 74 Kd spectrin variant, while in the present case which is clinically less severe, the proband is homozygous for a 65 Kd spectrin variant.

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REFERENCES

1. Cohen CM: The molecular organization of the red cell membrane skeleton. Semin Hematol 20:141, 1983

2. Palek J, Lux S: Red cell membrane skeletal defects in hereditary and acquired hemolytic anemia. Semin Hematol 20:189, 1985

3. Féo CJ, Fischer S, Piau JP, Grange MJ, Tchernia G: Première observation de l'absence d'une protéine de la membrane érythrocytaire (bande 4.1) dans un cas d'anémie elliptocytaire familiale. Nouv Rev Fr Hematol 22:315, 1980

4. Tchernia G, Mohandas N, Shohet SB: Deficiency of skeletal membrane protein band 4.1 in homozygous hereditary elliptocytosis. Implications for erythrocyte membrane stability. J Clin Invest 68:454, 1981

5. Alloisio N, Dorleac E, Girot R, Delaunay J: Analysis of the red cell membrane in a family with hereditary elliptocytosis. Total or partial absence of protein 4.1. Hum Genet 59:68, 1981

6. Garbarz M, Dhermy D, Lecomte MC, Féo C, Chaveroche I, Galand C, Bournier O, Bertrand O, Boivin P: A variant of erythrocyte membrane skeletal protein band 4.1 associated with hereditary elliptocytosis. Blood 64:1006, 1984

7. Alloisio N, Morlé L, Dorleac E, Gentilhomme O, Bachir D, Guetarni D, Colonna P, Bost M, Zouaoui Z, Roda L, Roussel D, Delaunay J: The heterozygous form of 4.1 (−) hereditary elliptocytosis (the 4.1 (−) trait). Blood 65:46, 1985

8. Liu SC, Palek J, Prchal JT: Defective spectrin dimer-dimer association in hereditary elliptocytosis. Proc Natl Acad Sci USA 79:2072, 1982

9. Dhermy D, Lecomte MC, Garbarz M, Bournier O, Galand C, Gautero H, Féo C, Alloisio N, Delaunay J, Boivin P: Spectrin β-chain variant associated with hereditary elliptocytosis. J Clin Invest 70:707, 1982

10. Lecomte MC, Dhermy D, Garbarz M, Gautero H, Bournier O, Galand C, Boivin P: Hereditary elliptocytosis with a spectrin molecular defect in a white patient. Acta Haemat 71:235, 1984

11. Lawler J, Liu SC, Palek J, Prchal J: A molecular defect of spectrin in a subset of patients with hereditary elliptocytosis. Alterations in the α-subunit domain involved in spectrin self-association. J Clin Invest 73:1688, 1984

12. Zail SS, Coetzer TL: Defective binding of spectrin to ankyrin in a kindred with recessively inherited hereditary elliptocytosis. J Clin Invest 74:753, 1984

13. Palek J: Hereditary Elliptocytosis and related disorders. Clinics Hematol 14:45, 1985

14. Chang K, Williamson JR, Zarkowski HS: Effect of heat on the circular dichroism of spectrin in hereditary pyropoikilocytosis. J Clin Invest 64:326, 1979

15. Liu SC, Palek J, Prchal J, Castleberry RP: Altered spectrin dimer-dimer association and instability of erythrocyte membrane skeleton in hereditary pyropoikilocytosis. J Clin Invest 68:597, 1981

16. Lawler J, Liu SC, Palek J: Molecular defect of spectrin in hereditary pyropoikilocytosis-alterations in the trypsin-resistant domain involved in spectrin self-association. J Clin Invest 70:1019, 1982

17. Dhermy D, Féo C, Garbarz M, Bournier O, Dommergues JP, Garcia J, Boivin P, Tchernia G: Anémie hémolytique congénitale par instabilité thermique érythrocytaire et défaut de tétramérisation de la spectrine. Nouv Rev Fr Hématol 25:7, 1983

18. Lawler J, Palek J, Liu SC, Prchal J, Butler WM: Molecular heterogeneity of hereditary pyropoikilocytosis: Identification of a second variant of the spectrin α-subunit. Blood 62:1182, 1983

19. Knowles WJ, Morrow JS, Speicher DW, Zarkowski HS, Mohandas N, Mentzer WC, Shohet SB, Marchesi VT: Molecular and functional changes in spectrin from patients with hereditary pyropoikilocytosis. J Clin Invest 71:1867, 1983

20. Agre P, Orringer EP, Bennett V: Deficient red-cell spectrin in severe, recessively inherited spherocytosis. N Engl J Med 306:1155, 1982

21. Wolfe LC, John KM, Falcone JC, Byrne AM, Lux SE: A genetic defect in the binding of protein 4.1 to spectrin in a kindred with hereditary spherocytosis. N Engl J Med 307:1367, 1982

22. Goodman SR, Shiffer KA, Casoria LA, Eyster ME: Identification of the molecular defect in the erythrocyte membrane skeleton of some kindreds with hereditary spherocytosis. Blood 60:772, 1982

23. Lux SE: Disorders of the red cell membrane skeleton: Hereditary spherocytosis and hereditary elliptocytosis, in Stranbury JB, Wyngaarden JB, Fredrickson DS, Goldstein JL, Brown SB (eds): The Metabolic Basis of Inherited Disease (5th ed). New York, Mc-Graw Hill, 1983, p 1573

24. Wyandt H, Bancroft PM, Winship TO: Elliptic erythrocytes in man. Arch Intern Med 68:1043, 1941

25. Lipton EL: Elliptocytosis with hemolytic anemia; the effects of splenectomy. Pediatrics 15:67, 1955

26. Grech JL, Cachia EA, Calleja F, Pullicino F: Hereditary elliptocytosis in two Maltese families. J Clin Pathol 14:365, 1961

27. Pryor DS, Pitney WR: Hereditary elliptocytosis: A report of 2 families from New Guinea. Br J Haematol 13:126, 1967

28. Evans JPM, Baines AJ, Hann IM, Al-Hakim I, Knowles SM, Hoffbrand AV: Defective spectrin dimer-dimer association in a family with transfusion dependent homozygous hereditary elliptocytosis. Br J Haematol 54:163, 1983

29. Dhermy D, Lecomte MC, Garbarz M, Feo C, Gautero H, Bournier O, Galand C, Herrera A, Grétillat F, Boivin P: Molecular defect of spectrin in the family of a child with congenital hemolytic poikilocytic anemia. Pediat Research 18:1005, 1984

30. Zarkowski HS, Mohandas N, Speaker CB, Shohet SB: A congenital haemolytic anaemia with thermal sensitivity of the erythrocyte membrane. Brit J Haematol 29:537, 1975

31. Bessis M, Mohandas N: Mesure continue de la déformabilité cellulaire par une méthode diffractométrique. CR Acad Sci (Paris) 278:3263, 1974

32. Groner W, Mohandas N, Bessis M: New optical technique for measuring erythrocyte deformability with the ektacytometer. Clin Chem 26:1435, 1980

33. Féo CJ, Nossal M, Jones E, Bessis M: Une nouvelle technique d'étude de la physiologie des globules rouges: La mesure de leur déformabilité en fonction de l'osmolalité. Résultats obtenus par un ektacytomètre automatisé sur le sang normal et dans différentes anémies hémolytiques. CR Acad Sci (Paris) 295:687, 1982

34. Litman D, Hsu CJ, Marchesi VT: Evidence that spectrin binds to macromolecular complexes on the inner surface on the red cell membrane. J Cell Sci 42:1, 1980

35. Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680, 1970

36. Fairbanks G, Steck TL, Wallach DFH: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606, 1971

37. O'Farrell P: High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007, 1975

38. Speicher DW, Morrow JS, Knowles WJ, Marchesi VT: A structural model of human erythrocyte spectrin. Alignment of chemical and functional domains. J Biol Chem 257:9093, 1982

39. Yip R, Mohandas N, Clark MR, Jain S, Shohet SB, Dallman PR: Red cell membrane stiffness in iron deficiency. Blood 62:99, 1983

40. Knowles WJ, Bologna ML, Chasis JA, Marchesi SL, Marchesi VT: Common structural polymorphisms in human erythrocyte spectrin. J Clin Invest 73:973, 1984

41. Lecomte MC, Dhermy D, Solis C, Ester A, Féo C, Gautero H, Bournier O, Boivin P: A new abnormal variant of spectrin in black patients with Hereditary Elliptocytosis. Blood 65:1208, 1985

42. Lawler J, Goetzer TL, Palek J, Jacob HS, Luban N: Sp $\alpha^{I/65}$: A new variant of the α subunit of spectrin in Hereditary Elliptocytosis. Blood 66:706, 1985

43. Dhermy D, Garbarz M, Lecomte MC, Féo C, Bournier O, Chaveroche I, Gautero H, Galand C, Boivin P: Hereditary elliptocytosis: Clinical, morphological and biochemical studies of 38 cases. Nouv Rev Fr Hematol (in press)

44. Mentzer WC, Turetsky T, Mohandas N, Schrier S, Chuen-Shang CW, Koenig H: Identification of the hereditary pyropoikilocytosis carrier state. Blood 63:1439, 1984

Myosin in Adult and Neonatal Human Erythrocyte Membranes

By Lisa M. Matovcik, Ute Gröschel-Stewart, and Stanley L. Schrier

The heavy chain of myosin can be detected in human red cell membranes by immunoblot analysis with antiplatelet myosin antibodies. Neonatal red cell membranes have more immunoreactive myosin than adult membranes. Membranes from young adult red cells contain more immunoreactive myosin than membranes from old adult red cells. In contrast, young and old neonatal red cells have equivalent amounts of myosin. Erythrocyte myosin is present in a membrane fraction enriched in integral membrane proteins but is not found in cytoskeletal preparations.
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THE RED cell membrane is flexible and deformable; its shape changes as it squeezes through capillaries in the microcirculation and recoils to a biconcave disc. It can pull in its echinocytic horns and invaginate or evaginate to form endocytic or exocytic vesicles. Before exiting the bone marrow the erythroid precursor expels the nucleus, thus becoming a motile reticulocyte.[1] Immature nucleated red cells internalize ligands necessary for cell growth by receptor-mediated endocytosis,[2-4] a process that involves myosin in other cell types.[5] The contractile proteins actin, myosin, and tropomyosin are present in the membrane.[6-8]

Myosin has been identified in and purified from human erythrocytes.[7] The erythrocyte myosin molecule consists of a 200 kDa heavy chain and two light chains of 25 kDa and 19.5 kDa.[7] It is present in small amounts, about 6,000 copies per red cell, less than half of which are bound to isolated membrane preparations.[7] Red cell membranes contain an actin-activated ATPase activity[9] which may correspond to myosin. In the platelet, a closely related cell type, phosphorylation of the 20 kDa myosin light chain results in an increase in the actin-activated ATPase activity.[10]

Neonatal red cell membranes are different from adult red cell membranes.[11] Neonatal but not adult red cells undergo easily visible levels of spontaneous endocytosis.[11-13] Drug-induced endocytosis is enhanced in neonatal compared to adult red cells.[14] The youngest circulating neonatal red cells undergo receptor-mediated endocytosis of transferrin, in contrast to an analogous cohort of young adult red cells.[15] Receptor-mediated endocytosis of Concanavalin A by neonatal but not adult red cells[16] occurs in spectrin-free zones in the membrane.[17]

Using an immunologic technique, the Western blot, these studies are the first to describe the presence of increased amounts of myosin in the neonatal compared to the adult red cell membrane. The amount of immunoreactive myosin was compared in young and old adult and neonatal red cells. Attempts were made to localize membrane myosin to either the cytoskeleton or to membrane fractions enriched in integral membrane proteins. Myosin is present both in the cytosol and the membrane, and the control of the amount of myosin that is associated with the membrane may be physiologically important. Therefore, the effects of the divalent cations Mg^{+2} and Ca^{+2} and red cell ATP levels on the amount of myosin present in the isolated membrane preparation were determined.

MATERIALS AND METHODS

Materials. All materials purchased were of reagent grade or better. Stractan was prepared as previously described.[14] Equipment for SDS-PAGE and Western blotting, nitrocellulose paper, and the chemicals acrylamide, bis-acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), and Temed were purchased from Biorad Laboratories (Richmond, Calif). Horseradish peroxidase conjugated swine antirabbit immunoglobulins and rabbit peroxidase-antiperoxidase were purchased from Dako Corporation (Santa Barbara, Calif) Swine serum was purchased from Gibco Laboratories (Grand Island, NY). The following reagents were purchased from Sigma Chemical Co (St Louis, Mo): alpha-cellulose, cellulose (Sigmacell-50), Tris-adenosine 5'-triphosphate, calcium ionophore A23187, Nonidet P-40 (NP40), 4-chloro, 1-napthol, and Triton X-100.

Methods. Protein purification and antibody production. Spectrin[18] and actin[9] were purified from outdated human erythrocytes. Protein 2.1[19] was purified from freshly drawn adult and neonatal blood. Platelet myosin was purified as described by Peleg et al.[20] Proteins were judged to be pure by SDS-PAGE electrophoresis.[21] Protein concentrations were determined either by the method of Lowry[22] or Bradford.[23]

Antibodies to platelet myosin were raised in rabbits using two platelet myosin preparations, platelet membrane myosin, and platelet cytoplasmic myosin.[20,24] These antibodies have been previously characterized.[25]

Erythrocyte membrane preparation and fractionation. Blood was drawn from normal adult volunteers and from the placental vessels of premature and term newborns at the time of delivery according to established protocols of the Stanford Committee for the Protection of Human Subjects in Research. Platelets were removed by defibrination, or removed along with the white cells by passage over a column of 50% cellulose, 50% alpha-cellulose.[26] This removed the white cells and platelets to below the threshold of detectability of the Coulter Model S Plus 4 or S Plus 2. Red cells were separated on discontinuous Stractan density gradients as previously described.[14] Red cell membranes were prepared by the method of Dodge.[27] Inside-out vesicles were prepared by the method of Steck and Kant,[28] and both isotonic and hypertonic Triton cytoskeletons by the method of Sheetz.[29]

Ca^{+2} loading and metabolic depletion. Calcium was introduced into adult and neonatal red cells with the calcium ionophore A23187. Red cells were incubated for 30 minutes at 37 °C at a hematocrit of approximately 0.15 in 0.154 mol/L NaCl with either 100 µmol/L $CaCl_2$ or 1.0 umol/L A23187 or both. In the ionophore controls, 1.0 mmol/L EDTA was added to ensure that small amounts of contaminating Ca^{+2} in the water would not enter RBC and confuse the results. Adult and neonatal red cells were depleted of ATP by gently

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rocking the whole blood in the sterile collection tubes in a horizontal position for 24 hours. After the incubations, the red cell ATP content was measured in an aliquot of cells,[30] an aliquot was fixed in glutaraldehyde and later examined by phase microscopy, and white ghosts were prepared from the rest of the red cells.

Electrophoresis and Western blotting. Gel electrophoresis was performed by the method of Laemmli[21] on convex gradient gels of 4% to 14% acrylamide with a 15 mL mixing volume. For antibody staining the gels were transferred at 50 volts for approximately 14 hours to nitrocellulose paper,[31] then incubated at 37 °C for 1 hour in 3% BSA, 1% swine serum (blocking solution). The primary antibody was diluted in blocking solution to a concentration of 50 to 100 μg protein/mL and incubated with the papers at 4 °C for 18 to 24 hours, washed extensively in PBS pH 7.4, then PBS with 0.05% NP40, again with PBS, then incubated with horseradish peroxidase labeled swine antirabbit immunoglobulin diluted 1:100 in blocking solution at room temperature for 90 minutes and the wash procedure repeated. The papers were stained with the chromagen 4-chloro, 1-napthol, 3 mg/mL in methanol (1 part), 154 mmol/L NaCl, buffered with 25 mmol/L Tris, pH 7.4 (5 parts), 3% H_2O_2 (0.2 parts). Color development was complete in 2 to 10 minutes.

ATPase assays. Mg^{+2}-ATPase and actin-activated ATPase assays were performed as previously described.[9] Ca^{+2}, Mg^{+2}-ATPase was assayed under conditions identical to those used for Mg^{+2}-ATPase with 120 μmol/L calcium chloride present. [Gamma-^{32}P]-ATP cleavage in all three assays was measured by the isobutanol-benzene method.[32]

RESULTS

Antibodies to human platelet myosin detect a 200K MW protein in the red cell membrane. Figure 1 shows that antiplatelet myosin cross-reacts with a 200 kDa protein in the red cell membrane, which comigrates with the platelet myosin heavy chain. The antibody does not bind to the platelet myosin light chains or any bands in the low molecular weight region of the red cell. The nonimmune immunoglobulin does not react with any of the proteins under study.

Two of the major proteins of the erythrocyte membrane, protein 2 (spectrin beta chain), and protein 2.1 (ankyrin), have a molecular weight similar to the myosin heavy chain and could obscure the myosin band on gels stained to visualise protein. The myosin heavy chain can be shown to be a protein separate from both proteins 2 and 2.1.[7] On 4% to 14% gradient gels transferred to nitrocellulose and stained with Amido Black, protein 2 is distinctly above the immunoreactive band on an identical paper that is stained with antiplatelet myosin. Likewise, overexposure of the antibody stained papers to the chromagen results in a faint background staining of all the proteins on the gel, and protein 2 can easily be distinguished from the darkly stained myosin. In order to determine if the antibody-stained protein is protein 2.1 we tested protein 2.1 purified from both adult and neonatal red cell membranes in the Western blot system and found that the purified protein 2.1 did not react with the antiplatelet myosin (data not shown).

Care was taken in the preparation of the red cells from which the ghosts were made to remove white cells and platelets, both of which contain myosin. The number of white cells and platelets remaining in the red cell preparations after filtration on the cellulose: alpha-cellulose column was always below the threshold of the electronic cell counter used to detect them. No white cells or platelets were visible upon microscopic examination of stained smears. We tested samples containing increasing, known numbers of platelets and white cells to determine the sensitivity of the antimyosin to contaminating cells under the Western blot conditions used in this study. The antiplatelet myosin can detect platelet myosin from a minimum of 2 × 10^4 platelets per gel lane.[33] This is three orders of magnitude more platelets than could have contaminated the 40 μg of ghost protein routinely applied to the gels given the manufacturer's stated detection threshold of the Coulter counter. Likewise, the number of white cells that contain the threshold of myosin the antibody is able to detect lies between 10 and 100 times the theoretical maximum number of white cells in the ghost sample loaded on the gel.

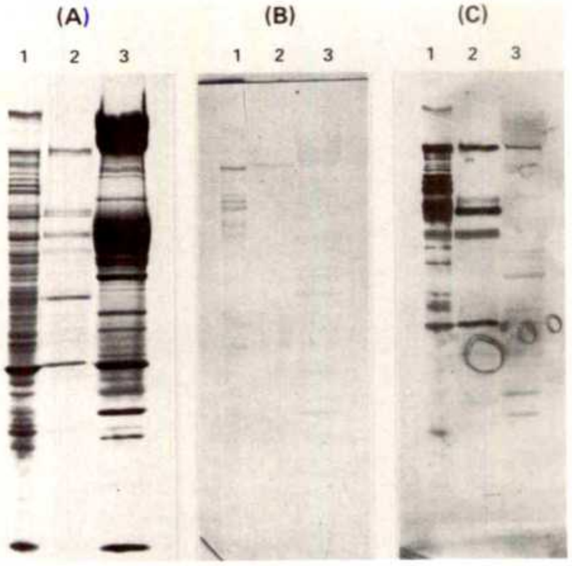

Fig 1. Identification of immunoreactive myosin in platelets and in the erythrocyte membrane. Samples containing 40 μg of protein of solubilized whole platelets (lanes 1), partially purified platelet myosin (lanes 2), and erythrocyte ghosts (lanes 3) were electrophoresed on gradient gels of 4% to 14% acrylamide. (A) was stained with Coomassie blue. (B) and (C) were transferred to nitrocellulose paper and incubated overnight at 4 °C with (B) 100 μg/mL nonimmune rabbit Ig and (C) with 100 μg/mL antiplatelet myosin Ig. Immunoreactive bands were detected by horseradish peroxidase labeled swine antirabbit Ig and stained with 4-chloro, 1-napthol.

Neonatal red cell membranes have greater reactivity to platelet antimyosin than adult red cell membranes. Figure 2 compares the antiplatelet myosin binding of white ghosts prepared from adult and neonatal red cells. Although there are no differences in the protein content of adult and neonatal red cells detectable by visual examination of Coomassie blue stained gels (panel A), the neonatal membrane contains more immunoreactive myosin than the adult membrane (panel B). Incubating adult and neonatal ghosts together at room temperature does not inhibit the antimyosin cross reactivity in the neonatal ghosts. The combination of adult and neonatal ghosts reacts with platelet antimyosin on a Western blot to approximately the same degree as the sum of the adult and neonatal membrane tested separately (data not shown).

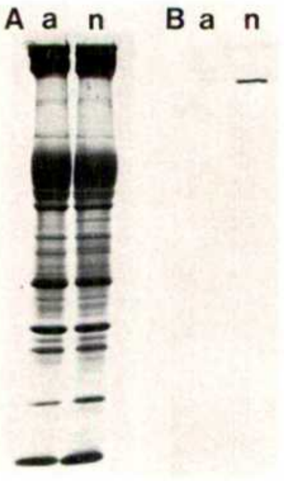

Fig 2. Comparison of the amount of immunoreactive myosin from white ghosts prepared from adult and neonatal erythrocytes. Samples containing 40 μg of white ghosts prepared from adult (a) or neonatal (n) erythrocytes were electrophoresed and treated as described in Fig 1. In (A) the gels were stained with Coomassie blue and in (B) with antiplatelet myosin as described in Fig 1.

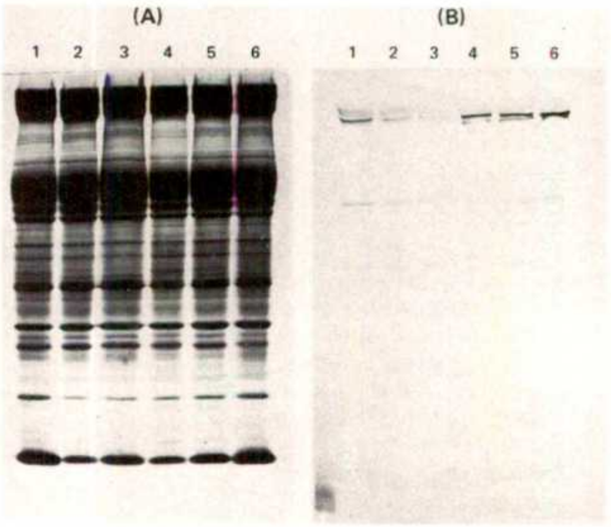

Fig 3. Density or age dependence of antimyosin reactivity in young, middle-aged, and old erythrocytes of adults and newborn infants. (A) Stained with Coomassie blue. (B) The corresponding antiplatelet myosin immunoblot. The electrophoresis and staining procedures are identical to those described in Fig 1. Ghosts prepared from young, middle-aged, and old red cells isolated from adults are shown in lanes 1 to 3 and from newborns in lanes 4 to 6. The young adult red cells in lane 1 and the young neonatal red cells in lane 4 have a density <1.0740 and comprise 2% of the adult and 13% of the neonatal red cell population. The old adult red cells in lane 3 and the old neonatal red cells in lane 6 have a density >1.0918 and comprise 1% of the adult and 4% of the neonatal red cells in this particular experiment. Note the predominance of protein 4.1b over 4.1a in the young neonatal ghosts in lane A4, which supports the idea that the lightest neonatal erythrocytes are a younger cell population than the lightest adult erythrocytes.

To determine whether enhanced antiplatelet myosin immunoreactivity is a feature of younger cells in general or only neonatal cells we separated both adult and neonatal red cells according to their density on discontinuous Stractan gradients.[14] In general, light cells are young and dense cells are old. Figure 3 shows the Coomassie blue stain, panel A, and the corresponding antiplatelet myosin Western blot, panel B, of young, middle-aged, and old white ghosts prepared from red cells isolated from adults (lanes 1–3), and newborns (lanes 4–6). In lane 1 (young), 2 (middle-aged), and 3 (old) adult red cells, the intensity of the antimyosin staining decreases with cell age. In lane 4 (young), 5 (middle-aged), and 6 (old) neonatal red cells, the intensity of antimyosin staining is greater in all three fractions than in the corresponding adult fraction. No decrease with age can be observed in neonatal red cells.

ATP depletion and increase in the intracellular Ca^{+2} level have no effect on the amount of myosin in the membrane. In order to determine conditions that might alter the amount of myosin that is bound to the membrane, we allowed Ca^{+2} to enter red cells with the ionophore A23187. In the presence of 100 μmol/L CaCl$_2$ and 1.0 μmol/L A23187, both the adult and neonatal red cells had a spheroechinocytic morphology.[34] This treatment reduced the ATP levels in these red cells to one half or less of their normal values (Table 1). Incubation of the red cells with 100 μmol/L-Ca^{+2} without ionophore did not result in an echinocytic shape change or lowered ATP levels.

Sterile incubation of whole blood at 37 °C for 24 hours depleted the red cell ATP content to barely detectable levels (Table 1). This incubation also resulted in extensive formation of echinocytes in both adult and neonatal red cells after depletion, with a few discocytes left. White ghosts were prepared from both Ca^{+2}-loaded and ATP-depleted red cells. The Western blot of the Ca^{+2}-loaded and ATP-depleted red cell membranes is shown in Fig 4. Neither treatment altered the amount of immunoreactive myosin present in either the adult or neonatal ghost preparation.

Effect of Mg^{+2} and Mg^{+2}-ATP on the membrane-associated myosin. The addition of 1.0 mmol/L MgCl$_2$ to the white ghost lysis buffer results in more tropomyosin associating with the membrane preparation.[8] To determine whether Mg^{+2} resulted in more myosin association as well, white ghosts were prepared with 1.0 mmol/L MgCl$_2$ added to the lysis buffer (5 mmol/L PO$_4$, pH 8.0). No difference in the intensity of antimyosin staining was detected as a result (data not shown).

Since ATP affects the association of actin with myosin, we studied the effect of adding Mg^{+2}-ATP to the lysis buffer. White ghosts were prepared with 1 mmol/L MgCl$_2$ and 1 mmol/L Tris-ATP in the lysis buffer, and each of the three washes. Western blot analysis with anti-platelet myosin did not detect any differences between ghosts prepared with or without Mg^{+2}-ATP (Fig 4, Panels A & B, lanes 2).

Myosin is present in inside-out vesicles, but not in Triton cytoskeletons. In order to localize the membrane domain that contains myosin we fractionated the red cell membrane into inside-out vesicles[28] and Triton cytoskeletons[29] (see Methods). Inside-out vesicles (IOVs) are depleted in cytoskeletal proteins and enriched in integral membrane proteins such as protein 3 and the glycophorins (Fig 5A, lanes 2 and

Table 1. ATP Levels in Ca^{+2}-Loaded and ATP-Depleted RBC

	ATP (μmol/mL RBC)	
	Adult	Neonate
Untreated RBC	1.80	1.64
CaCl$_2$ + A23187	0.61	0.80
Sterile depletion	0.01	0.06

6). Triton cytoskeletons are enriched in the cytoskeletal proteins spectrin, actin, and protein 4.1. When isolated under isotonic conditions, Triton cytoskeletons also contain small amounts of various other membrane proteins (Fig 5A, lanes 3 and 7),[29] but Triton cytoskeletons isolated under hypertonic conditions (0.6 M KCl) retain very little of proteins 3, 2.1, or 4.2, glycophorins, or other minor membrane components and are composed almost solely of spectrin, actin, and protein 4.1 (Fig 5A, lanes 4 and 8). The presence of myosin was monitored by Western blot analysis and an assay for actin-activated ATPase activity was performed on each fraction.

The Western blot analysis with antiplatelet myosin is shown in Fig 5B. The red cell membrane myosin remains with the inside-out vesicles and is not present in Triton cytoskeletons prepared either isotonically or hypertonically.

ATPase activities of adult and neonatal red cell membranes. Red cell ghosts contain several well-characterized ATPase activities,[9,36] among them an actin-activated Mg^{+2}-ATPase which is an identifying characteristic of myosin. We compared the activity of Mg^{+2}-ATPase, actin-activated Mg^{+2}-ATPase, and Ca^{+2}, Mg^{+2}-ATPase in adult and neonatal red cells (Table 2) to see if the enzyme activities correspond to the degree of antimyosin reactivity. If myosin is present in the isolated membrane preparation along with the regulatory factors necessary for activity, then the neonatal red cell membrane would be expected to have more actin-activated Mg^{+2}-ATPase activity than adult membranes.

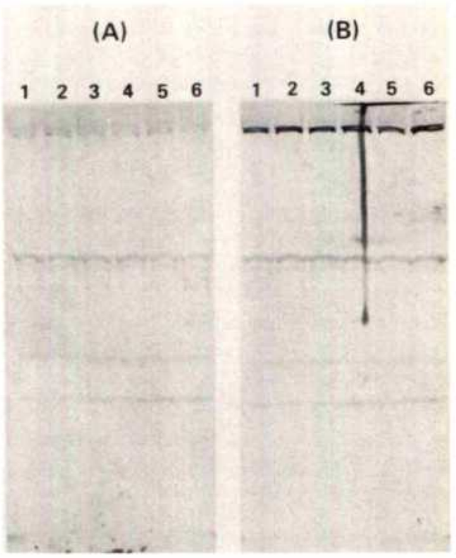

Fig 4. Western blots of white ghosts from adult (A) and neonatal (B) RBC, stained as in Fig 1 with antiplatelet myosin. Lanes 1 are control ghosts, lanes 2 are ghosts prepared with 1 mmol/L Mg^{+2}-ATP added to the lysis buffer. Lanes 3 are ghosts prepared from RBC treated with Ca^{+2}. Lanes 4 are prepared from RBC treated with ionophore and EDTA, and lanes 5 are prepared from RBC treated with ionophore and Ca^{+2}. Lanes 6 are prepared from ATP-depleted RBC. There is a dye artifact in B4.

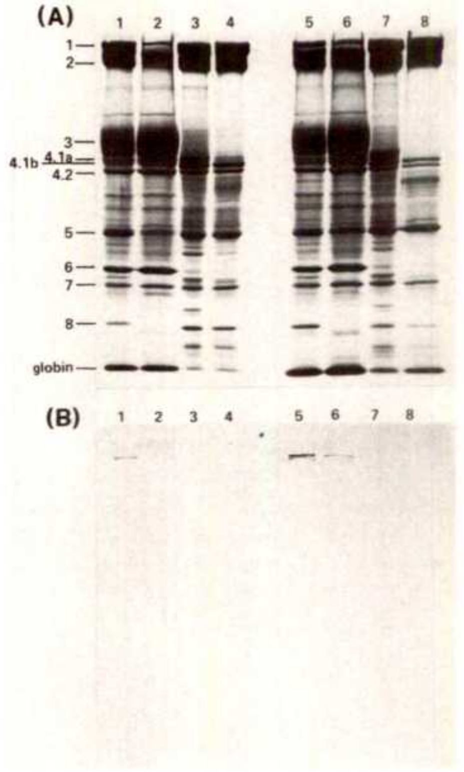

Fig 5. Localization of myosin in the IOVs of erythrocyte membranes. Samples containing 40 μg of protein per lane were electrophoresed on gradient gels of 4% to 14% acrylamide. Lanes 1 to 4 are adult membrane fractions while lanes 5 to 8 are neonatal membrane fractions. (A) Stained with Coomassie blue. (B) Transferred to nitrocellulose paper and incubated overnight at 4 °C with 100 μg/mL platelet antimyosin Ig. Lanes 1 and 5 contain white ghosts. Lanes 2 and 6 contain inside-out vesicles (IOVs).[28] Lanes 3 and 7 contain Triton cytoskeletons prepared from red cells in isotonic buffer. Lanes 4 and 8 contain Triton cytoskeletons prepared from red cells in buffer containing 0.6 mol/L KCl. The nomenclature is that of Fairbanks.[36]

Table 2 compares the Mg^{+2}, actin-activated, and Ca^{+2}, Mg^{+2}-ATPase activities of adult and neonatal red cell membranes. There is no significant difference between the baseline level of total Mg^{+2}-ATPase in freshly prepared neonatal and adult white ghosts. This Mg^{+2}-ATPase activity is age-

Table 2. ATPase Activities of Adult and Neonatal Erythrocyte Ghosts

	Adult	Neonate
Mg^{+2}-ATPase	754 ± 248 (n = 12)	697 ± 219 (n = 18)
Actin-activated Mg^{+2}-ATPase	442 ± 457 (n = 8)	710 ± 515 (n = 5)
Ca^{+2}, Mg^{+2}-ATPase	517 ± 186 (n = 8)	653 ± 268 (n = 23)

White ghosts were assayed as described in Methods: ATPase activities are expressed as nmol P$_i$ hydrolyzed from ATP/mg protein/hr (mean ± SD).

( ) number of separate analyses performed.

dependent; ghosts from young red cells have more activity than ghosts from old cells. The mean population of neonatal red cells has the same amount of Mg^{+2}-ATPase activity as adult red cells but the age dependence is more pronounced between the youngest and oldest adult cells (data not shown). Addition of erythrocyte F-actin (but not rabbit muscle F-actin) results in an increase in a component of the Mg^{+2}-ATPase activity to approximately the same extent in neonatal and adult red cells. Actin-activated Mg^{+2}-ATPase shows no age dependence in adult or neonatal red cells. The Ca^{+2}, Mg^{+2}-ATPase activity does not differ significantly in adult and neonatal red cell membranes.

Actin-activated Mg^{+2}-ATPase activity was measured in ghosts, IOVs, and Triton cytoskeletons and compared to the amount of immunoreactive myosin. Although IOVs retain 56% and Triton cytoskeletons 12% of the total Mg^{+2}-ATPase activity of ghosts per weight membrane protein, neither membrane fraction has any measurable actin-activated Mg^{+2}-ATPase activity.

The myosin immunoreactivity and actin-activated ATPase activity do cosediment when ghosts are extracted with 0.6 mol/L KCl, a method used to extract myosin from smooth muscle and nonmuscle cells. Red cell myosin is not soluble in 0.6 mol/L KCl. The supernatant neither contains actin-activated ATPase activity nor reacts with antiplatelet myosin in the Western blot system. The pellet does contain immunoreactive myosin, and the actin-activated ATPase activity can be recovered quantitatively in this fraction (data not shown).

An experiment testing whether antiplatelet myosin could inhibit the actin-activated ATPase activity of ghosts was not conclusive because, although equimolar amount of immunoglobulin to membrane protein did effectively eliminate ATPase activity in leaky ghosts, the control antibody to a cytosolic-facing membrane protein, spectrin, had the same effect.

DISCUSSION

Myosin has only recently been identified in the human red cell,[7,37] probably because it is present in very small amounts, and because human red cell membrane myosin requires an extraction procedure that differs from methods routinely used to purify uterine and platelet myosin.[20,38] Tryptic peptide analysis shows that erythrocyte myosin is very similar to platelet myosin.[7]

In this report we relied on immunoblot analysis to establish the presence or absence of red cell membrane myosin and to indicate the relative amount present. While it would have been preferable to do a chemical extraction and purification, such experiments are not technically possible because of the small amount of myosin present in the red cell: about 6,000 copies per cell.[7,37] The amounts of blood required (1 to 2 units)[7,37] cannot be obtained at all from neonates, and would be difficult to obtain from density-separated populations of red cells and from fractions of integral membrane proteins and cytoskeletal preparations.

Our results (Figs 2, 3, 4, and 5) show that more immunoreactive myosin is present in the membranes of neonatal than in adult red cells. The antiplatelet membrane myosin gave the same results as anticytoplasmic myosin although the two immunogens are distinct.[25] Neonatal red cells may contain more myosin or more of the total red cell myosin may be associated with the membrane. Erythrocyte myosin may behave similarly to platelet[39] or brush border epithelial cell[40] myosin and be present in an equilibrium between the cytoplasm and the membrane which depends on its state of phosphorylation. A third possibility is that there is a fetal myosin isozyme that is more immunoreactive with these particular antibodies.

Since neonatal erythrocytes are in general a younger cell population than adult erythrocytes, we determined the contribution of cell age by separating adult and neonatal red cells into density, and thus probably age-dependent, populations and compared the amount of antiplatelet myosin reactivity in each. The amount of immunoreactive membrane myosin decreases as the adult erythrocyte ages. Immature red cells remodel their membranes to a greater degree than mature red cells; myosin may be involved in this remodeling. No decrease in immunoreactivity with age was observed in neonatal red cell membranes. The interpretation of this result is complicated by the fact that the older neonatal red cells were produced by a less developmentally mature fetus than the younger neonatal red cells.

The amount of red cell membrane-associated myosin may be controlled by physiologic events as it is in other tissues. Differences in these control mechanisms could account for the differences in immunoreactive myosin seen in adult and neonatal red cell membranes. In other tissues the amount of Ca^{+2}, Mg^{+2}, or ATP can influence the extent of membrane association by influencing the state of myosin phosphorylation. However, neither ATP depletion nor Ca^{+2} accumulation nor Mg^{+2}-ATP addition to lysis buffer altered membrane myosin retention in adult or neonatal RBC membranes (Fig 4).

Since actin is an important component of the red cell membrane cytoskeleton, we assumed that cytoskeletal preparations would be enriched in immunoreactive myosin as well, whereas the inside-out vesicles, stripped of their skeletal proteins and containing the integral transmembrane proteins, would have little myosin. In fact, the opposite was the case, and equally so in neonatal and adult membrane preparations (Fig 5).

Actin-activated ATPase activity, which serves to identify myosin, is present in red cell ghosts. Estimates of the number of molecules of myosin per red cell, and the percent of myosin that is membrane-associated[7,37] suggest that about 1/1000 of the adult red cell membrane protein weight is myosin. The Mg^{+2}-ATPase activity stimulated by human erythrocyte actin can then be calculated to be roughly 8 μmol P_i/mg myosin/min, a value considerably higher than results obtained using rabbit muscle actin.[37] However in our prior study we found that rabbit muscle actin induced very little actin-activated MG^{+2}- ATPase activity compared to human erythrocyte actin. We then sought to determine if this enyzme activity parallels the immunoblot data. Both antimyosin reactivity and actin-activated Mg^{+2}-ATPase were present in the residual pellet of a 0.6 mol/L KCl extraction, but the enzyme activity was lost in the IOV and the Triton cytoskeleton preparations. If antiplatelet myosin reactivity and actin-activated Mg^{+2}-ATPase activity are detecting the same protein, then modulators of the enzyme activity could be lost when the membrane is disrupted. The ATPase of purified nonmuscle myosins is not activated by actin unless necessary cofactors such as myosin light chain kinase are present.[41] For example, platelet[10] and macrophage[42] myosin are both regulated by phosphorylation of the 20 kDa light chain by a myosin light chain kinase. Neonatal ghosts and IOVs contain more immunoreactive myosin than adult ghosts and IOVs but do not have substantially more actin-activated Mg^{+2}-ATPase activity. The reasons for this discrepancy may become clear when the regulation of enzymic activity of erythrocyte myosin is understood.

The discovery of myosin in red cells[7,37] establishes the fact that a potentially complete contractile system, including myosin, actin, and tropomyosin, is present at the membrane. The role of the contractile apparatus is currently unknown. Very likely it is functional in motile red cell progenitors[1] where it may be involved actively in nuclear extrusion or receptor-mediated endocytosis. Reticulocytes are formed when the orthochromic normoblast ejects the nucleus with dynamic contractions.[43] Erythroblasts undergo receptor-mediated endocytosis to internalize ligands necessary for cell growth. Contraction of the actomyosin complex has recently been implicated in insulin receptor endocytosis in human lymphoblastoid cells.[5] The fact that myosin is found associated with integral red cell membrane protein in IOVs and not with actin in the cytoskeleton also raises the hypothesis that under certain unknown circumstances the interaction between myosin and actin could serve to link cytoskeletal components with integral proteins in a manner that supplements the spectrin-ankyrin-protein 3 interaction.

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REFERENCES

1. Mel HC, Prenant M, Mohandas N: Reticulocyte motility and form: Studies on maturation and classification. Blood 49:1001, 1977

2. Hemmaplardh D, Morgan E: The role of endocytosis in transferrin uptake by reticulocytes and bone marrow cells. Brit J Haematol 36:85, 1977

3. Harding C, Heuser J, Stahl P: Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. J Cell Biol 97:329, 1983

4. Iacopetta B, Morgan E, Yeoh G: Receptor mediated endocytosis of transferrin by developing erythroid cells from the fetal rat liver. J Histochem Cytochem 31:336, 1983

5. Majercik M, Bourguignon L: Insulin receptor capping and its correlation with Ca^{+2} and myosin light chain kinase. J Cell Biol 99:208a, 1984 (abstr)

6. Ohnishi T: Extraction of actin and myosin-like proteins from erythrocyte membranes. J Biochem (Tokyo) 52:307, 1962

7. Fowler VM, Davis JQ, Bennett V. Human erythrocyte membrane myosin: Identification and purification. J Cell Biol 100:47, 1985

8. Fowler VM, Bennett V: Erythrocyte membrane tropomyosin. J Biol Chem 259:5978, 1984

9. Schrier SL, Hardy B, Junga I, Ma L: Actin activated ATPase in human red cell membranes. Blood 58:953, 1981

10. Adelstein RS, Conti MA: Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. Nature 256:597, 1975

11. Matovcik L, Mentzer W: The membrane of the human neonatal red cell, in Schrier S (ed): Clinics in Haematology (vol 14). London, W.B. Saunders, 1985, p 203

12. Haberman S, Blanton P, Martin J: Some observations on the ABO antigen sites of the erythrocyte membranes of adults and newborn infants. J Immunol 98:150, 1967

13. Holroyde C, Oski F, Gardner F: The "pocked" erythrocyte. Red-cell surface alterations in the reticuloendothelial immaturity of the neonate. N Engl J Med 281:516, 1969

14. Matovcik LM, Junga IG, Schrier SL. Drug induced endocytosis of neonatal erythrocytes. Blood 65:1056, 1985

15. Matovcik LM: The membrane of the human neonatal red blood cell (PhD thesis), 1985, p 74

16. Schekman R, Singer S: Clustering and endocytosis of membrane receptors can be induced in mature erythrocytes of neonatal but not adult humans. Proc Natl Acad Sci USA 73:4075, 1976

17. Tokuyasu K, Scheckman R, Singer S: Domains of receptor mobility and endocytosis in the membranes of neonatal human erythrocytes and reticulocytes are deficient in spectrin. J Cell Biol 80:481, 1979

18. Fuller G, Boughter J, Morzzani M: Evidence for multiple polypeptide chains in the membrane protein spectrin. Biochem 13:3036, 1974

19. Tyler JM, Hargreaves WE, Branton D. Purification of two spectrin-binding proteins: Biochemical and microscopic evidence for site specific reassociation between spectrin and bands 2.1 and 4.1. Proc Natl Acad Sci USA 76:5192, 1979

20. Peleg I, Kahane I, Eldor A, Gröschel-Stewart U, Mestan J, Muhlrad A: Structural properties of myosin from the particulate fraction of human blood platelets. J Biol Chem 258:9290, 1983

21. Laemmli U: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

22. Lowry O, Rosebrough N, Farr A, Randall R: Protein measurements with the folin phenol reagent. J Biol Chem 193:265, 1951

23. Bradford M: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248, 1976

24. Peleg I, Muhlrad A, Eldor A, Gröschel-Stewart U, Kahane I: Characterization of the ATPase activities of myosins isolated from the membrane and the cytoplasmic fractions of human platelets. Arch Biochem and Biophy 234:442, 1984

25. Gröschel-Stewart U, Rakowsky C, Franke R, Peleg I, Kahane I, Eldor A, Muhlrad A: Immunohistochemical studies with antibodies to myosins from the cytoplasm and membrane fraction of human platelets. Cell Tissue Res 241:399, 1985

26. Beutler E, West C, Blume K-G: The removal of leukocytes and platelets from whole blood. J Lab Clin Med 88:328, 1976

27. Dodge J, Mitchell C, Hanahan D: The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. Biochem Biophys Acta 100:119, 1963

28. Steck T, Kant J: Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. Meth Enzymol 31:172, 1974

29. Sheetz M, Sawyer D: Triton shells of intact erythrocytes. J Supramolecular Structure 8:399, 1978

30. Beutler E: Red Cell Metabolism. Orlando, Fla, Grune and Stratton, 1975, p 104

31. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76:4350, 1979

32. Schrier S, Giberman E, Danon D, Katchalski E: Studies on ATPase in sheared microvesicles of human erythrocyte membranes. Biochim Biophys Acta 196:263, 1970

33. Pollard T, Kujiwara K, Handin R, Weiss G: Contractile proteins in platelet activation and contraction. Ann NY Acad Sci 283:218, 1977

34. Palek J, Liu S-C: The dependence of spectrin organization in red blood cell membranes on cell metabolism: Implications for control of red cell shape, deformability, and surface area. Sem Hematol 16:75, 1979

35. Fairbanks G, Steck T, Wallach D: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem 10:2606, 1971

36. Drickamer K: The red cell membrane contains three different adenosine triphosphatases. J Biol Chem 250:1952, 1975

37. Wong A, Kiehart D, Pollard T: Myosin from human erythrocytes. J Biol Chem 260:46, 1985

38. Perry SV: Myosin adenosine-triphosphatase, in Colowick SP, Kaplan NO (eds): Methods in Enzymology II. Orlando, Fla, Academic Press, 1955, p 582

39. Fox J, Phillips D: Role of phosphorylation in mediating the association of myosin with the cytoskeletal structure of human platelets. J Biol Chem 257:4120, 1982

40. Broschat K, Stidwell R, Burgess D: Phosphorylation controls brush border motility by regulating myosin structure and association with the cytoskeleton. Cell 35:561, 1983

41. Mooseker M, Pollard T, Kujiwara K: Characterization and localization of myosin in the brush border of intestinal epithelial cells. J Cell Biol 79:444, 1978

42. Trotter J, Adelstein R: Macrophage myosin: Regulation of actin activated ATPase activity by phosphorylation of the 20,000 dalton light chain. J Biol Chem 254:8781, 1979

43. Bessis M, Lessin L, Beutler E: Morphology of the erythron, in Williams W, Beutler E, Erslev A, Lichtmann M (eds): Hematology. New York, McGraw-Hill, 1983, p 264

Lipoxygenase Metabolites of Arachidonic Acid Modulate Hematopoiesis

By David S. Snyder and Jane F. Desforges

Lipoxygenase (LPO) metabolites of arachidonic acid participate in the activation and/or proliferation of a variety of cell types. In this study, we examined the role of LPO metabolites in controlling myelopoiesis and erythropoiesis in vitro. Monocyte depleted cells (MDC) prepared from human whole blood or whole mononuclear cells from human bone marrow were cultured in methylcellulose in the presence of various growth factors. Conditioned media containing human colony stimulating factors (CSF) or the tumor-promoting phorbol ester, phorbol myristate acetate (PMA), were added to induce myelopoiesis. Semipurified human erythropoietin (EPO) was added along with an endogenous source of burst-promoting activity (BPA) to induce erythropoiesis. The LPO inhibitor BW755C blocked all types of colony formation in a dose-dependent manner, with ID_{50} of 20 and 5 μg/mL for myeloid and erythroid colonies, respectively. MDC depleted of T cells were similarly inhibited by BW755C. Similar results were seen with two other LPO inhibitors, 1-phenyl-3-pyrazolidone and butylated hydroxyanisole. A fourth LPO inhibitor, nordihydroguaiaretic acid, inhibited at higher concentrations. Indomethacin, at concentrations that inhibit cyclooxygenase, had no significant effect, either alone or in combination with the LPO inhibitors. These results suggest that certain LPO products may be important mediators of both CSF- and PMA-induced myelopoiesis, and of BPA/EPO-induced erythropoiesis.

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MYELOID and erythroid progenitor cells, known as CFU-GM and BFU-E, respectively, form colonies in vitro only when cultured in the presence of specific growth factors.[1,2] CFU-GM, as well as more mature cells of myeloid lineage, bear cell surface receptors for a family of glycoproteins called colony-stimulating factors (CSF).[1,3,4] Some of these receptors may have tyrosine kinase activity.[5] Various CSF promote colonies that consist of either granulocytes, monocyte/macrophages, eosinophils, or combinations thereof.[1] In addition, tumor-promoting phorbol esters such as phorbol myristate acetate (PMA) induce monocytopoiesis, while inhibiting granulopoiesis.[6-9] PMA appears to bind to target cells at a site different from the CSF receptor,[9] then activates protein kinase C,[10] and possibly also a tyrosine kinase.[11] Little is known about the receptors for burst-promoting activity (BPA), and erythropoietin (EPO) presumably present on BFU-E and the more mature erythroid progenitor cells, CFU-E.[12]

The intracellular events triggered by these ligand-receptor interactions that lead to cell proliferation and differentiation are poorly understood. Some data suggest that the arachidonic acid (AA) pathway is important in modulating these responses. First corticosteroids, which block the release of AA from membrane phospholipids,[13] and prostaglandin E, a cyclooxygenase (CO) product, inhibit the myelopoietic effects of CSF[14,15] and PMA.[15] Second, PMA stimulates production of both CO and lipoxygenase (LPO) products of AA in various target cells.[16,17] Products of the LPO pathway in particular have been implicated as critical intermediates in the activation and/or proliferation of a variety of cell systems including acute inflammation,[18,19] hormone secretion,[20-22] and cell-mediated immune responses.[23-27]

In this study we examined the role of LPO products in regulating hematopoiesis by observing the effects of LPO inhibitors on the response of CFU-GM and BFU-E to their respective growth factors. We compared the effects of four compounds known to inhibit both lipoxygenase and cyclooxygenase enzymes[27-30] with those of indomethacin at a concentration known to inhibit only CO activity.[28,31] Any differences in the results would therefore be attributed to the actions of LPO metabolites (see Fig 1).

MATERIALS AND METHODS

Preparation of cells

Peripheral blood was collected from normal male donors in preservative-free heparin. Bone marrow aspirates from patients with apparently normal myelopoiesis and/or erythropoiesis were collected in heparinized syringes after verbal consent was given. The whole mononuclear cell fraction (WMNC) was separated from whole blood or bone marrow by Ficoll-Hypaque density centrifugation. Bone marrow WMNC were washed three times in alpha minimal essential medium (α-MEM), then plated as described below without further processing. WMNC from whole blood were washed three times in α-MEM, then incubated in plastic petri dishes containing α-MEM with 20% human AB serum at 37 °C, 5% CO_2, for 1 to 2 hours to remove adherent cells. Nonadherent cells were removed and incubated with carbonyl iron (Lymphocyte Separator Reagent, Technicon) at 37 °C for 30 minutes, then layered on Ficoll-Hypaque to remove phagocytic cells. Monocyte-depleted cells (MDC) were recovered from the interface and washed twice with α-MEM before plating. MDC contained less than 1% monocytes by Wright-Giemsa and nonspecific esterase staining, and were greater than 95% viable by trypan blue dye exlusion. To remove T cells, the cells were incubated overnight with sheep cells and the rosetted cells removed by density centrifugation. The cells were then incubated again for one hour with sheep cells treated with 2-aminoethylisothiouronium (AET), and the remaining T cells rosetted and removed by density centrifugation.[32] The final preparation contained <5% T cells.

Progenitor Cell Assays

Myeloid progenitor cell (CFU-GM) assays contained 1 mL of NCTC 109 media with 30% heat-inactivated fetal calf serum and

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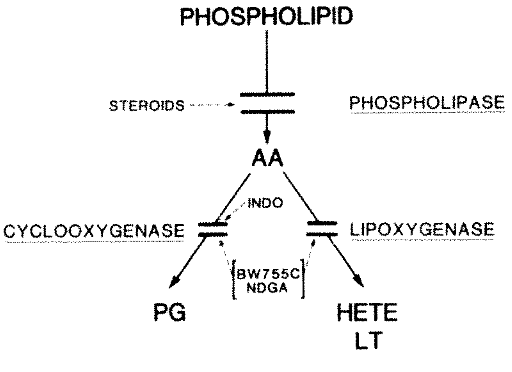

Fig 1. Inhibitors used in this study. Inhibition is denoted by dashed arrows and double bars. Not shown are 1-phenyl-3-pyrazolidone and butylated hydroxyanisole, which act at the same sites as BW755C and NDGA. AA, arachidonic acid; PG, prostaglandins, thromboxane, and prostacyclin; HETE, hydroxyeicosatetraenoic acids; LT, leukotrienes.

0.9% methylcellulose. In most experiments Gibco Giant Cell Tumor–Conditioned Media (GCT-CM) at 5% vol/vol, which provided maximal growth stimulation, was added to the cultures as the source of colony-stimulating activity. In other experiments PMA (Sigma) at various concentrations or 5% vol/vol human placenta–conditioned media was added as the stimulus for colony formation.

Assays for immature erythroid progenitor cells (BFU-E) contained 1 mL of NCTC 109, 30% fetal calf serum, 1% bovine serum albumin, 10^{-4}mol/L β-mercaptoethanol, 1.5 × 10^{-4}mol/L L-asparagine, 2 units of human urinary erythropoietin (CAT 1, kindly provided by the National Heart, Lung and Blood Institute), and 0.9% methylcellulose.

Whole blood MDC were plated in duplicate at 10^6 cells per mL, and bone marrow WMNC at 2 × 10^5 cells per mL at 37 °C, 5% CO_2 in humidified air. CFU-GM colonies (aggregates of greater than 40 cells) were counted after 7 days for bone marrow WMNC and after 14 days for whole blood MDC using an inverted microscope. In some experiments, clusters (10 to 40 cells) were also counted. Cytospin preparations of plucked CFU-GM colonies were stained with Wright-Giemsa or nonspecific esterase to confirm the presence of granulocytes, monocytes/macrophages, and eosinophils. BFU-E from both bone marrow and blood were counted after 14 days. They were identified as progenitors of large single colonies of greater than 50 hemoglobinized cells or clusters of colonies in typical burst formation.

Liquid Cultures

In some experiments MDC were incubated for 24 to 48 hours in NCTC 109 containing 10% fetal calf serum at 37 °C, 5% CO_2, before plating them in methylcellulose.

Drugs

The inhibitors used in this study were (1) BW755C (a generous gift of Wellcome Research Laboratories), (2) nordihydroguaiaretic acid (NDGA, Sigma, St Louis), (3) 1-phenyl-3-pyrazolidone (1P3P, Sigma), (4) butylated hydroxyanisole (BH, Sigma), and (5) indomethacin (Sigma). The first four compounds are inhibitors of both lipoxygenase and cyclooxygenase enzymes,[29–31,33] whereas indomethacin, used at 10^{-6}mol/L, inhibits only cyclooxygenase activity.[33,34] These drugs were dissolved in absolute ethanol, then diluted with aqueous media to the desired concentration. The maximum final ethanol concentration of 0.1% had no effect on colony growth.

RESULTS

Myelopoiesis

Effects of GCT-CM. In the absence of conditioned media, the background number of colonies grown from whole blood MDC was 30 ± 16 (mean ± SEM) per 10^6 cells plated. The number of colonies that grew in response to GCT-CM was 110 ± 18 per 10^6 cells plated. By colony morphology and by Wright-Giemsa staining of plucked colonies, there was a mixture of eosinophil, granulocyte-macrophage, and mixed colonies. Indomethacin at 10^{-6}mol/L had no effect on either the background or positive control responses.

Fig 2 shows that BW755C inhibited formation of CFU-GM colonies derived from MDC in a dose-dependent manner, with ID_{50} of 18 μg/mL. Cluster formation (aggregates of 10 to 40 cells) and background colony growth were inhibited in a parallel manner. All types of colonies were inhibited equally. This inhibition could not be overcome by increasing the concentration of GCT-CM. Furthermore, similar results were obtained using HPCM instead of GCT-CM, or using bone marrow WMNC instead of whole blood MDC. 1P3P and BH completely inhibited the response of blood MDC to GCT-CM with ID_{50} of 50 μg per mL and 20 μg per mL, respectively (see Table 1). The dose response to NDGA is shown in Fig 3. At concentrations greater than 5 × 10^{-6}mol/L, NDGA inhibited colony formation in a dose-dependent manner. The slight increase at lower concentrations is not statistically significant.

In order to determine whether the inhibitors may be acting indirectly on progenitor cells by an effect on regulator cells, experiments were carried out with culture of a cohort of cells depleted of T cells as well as monocytes by double adherence and double rosetting procedures. Four experiments were carried out, and in all a dose response curve of increasing inhibition was noted with the addition of BW755C (see

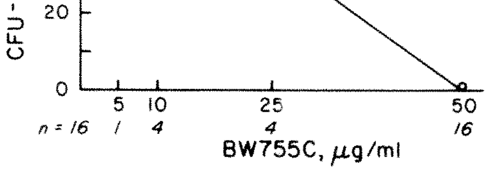

Fig 2. Inhibition of CSF by BW755C. GCT-CM, as the source of CSF, was added along with BW755C on day 0 of 14-day cultures of blood MDC. Results are expressed as percent of control, which was normalized to 100% within each experiment. n, number of experiments represented. Each point represents the mean ± SEM in this and in subsequent figures. See Results for absolute numbers of background and control colonies.

Table 1. Effect of LPO Inhibitors on CFU-GM Growth

Inhibitor µg/mL 1P3P	Inhibitor µg/mL BH	CFU-GM Colonies % Control
—	—	100
5	—	100
20	—	69
50	—	9
100	—	0
—	5	74
—	10	63
—	25	41
—	50	0
—	100	0

GCT-CM was added on day 0 along with either 1P3P or BH at the indicated concentrations to 14-day cultures of blood MDC. The data represent the means of duplicate samples combined from two representative experiments.

Table 2). Indomethacin, 10^{-6}mol/L, had no effect on the inhibition of colony growth by the LPO inhibitors.

To test whether the LPO inhibitors were nonspecifically toxic to progenitor and/or accessory cells, we measured the effects of these drugs on cell viability and the reversibility of inhibition. In Table 3, MDC were incubated with or without BW755C for 24 hours in liquid suspension. The cells were then washed three times in fresh media and plated in methylcellulose containing GCT-CM. There was no difference in the number of colonies formed after preincubation with BW755C compared to the control. In contrast, addition of BW755C to methylcellulose at the start of the 14-day culture completely blocked colony formation.

Effects of phorbol myristate acetate. Monocyte colony formation is stimulated by PMA and related tumor-promoting phorbol esters.[9] To assess whether PMA-induced monocytopoiesis depends on LPO metabolites, as CSF-induced myelopoiesis appears to do, we evaluated the response to PMA in our system. Figure 4 shows that PMA had complex effects on MDC-derived CFU-GM colonies. At 10^{-8}mol/L, PMA completely inhibited eosinophil and granulocyte colony growth, though the total number of colonies was the same as the control. At 10^{-7}mol/L, PMA again inhibited eosinophil and granulocyte colonies, but stimulated the total number of colonies over background. These colonies appeared to consist of monoblasts and monocytes by Wright-Giemsa and nonspecific esterase staining. In subsequent experiments PMA was used at 10^{-7}mol/L.

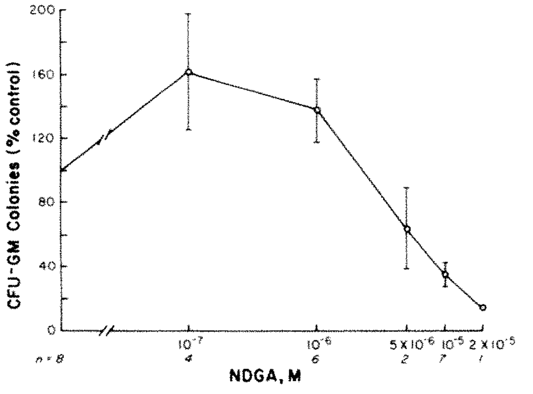

Fig 3. Effect of NDGA on CSF. See Fig 2 legend for details.

Table 2. Effect of BW 755C on Progenitor Cells Depleted of Monocytes and T Cells

Expt	BW 755C µg/mL 0	5	10	25	50
1	100	77	38	0	0
2	100	74	43	4	0
3	100	66	34	0	0
4	100	73	36	4	0

Data represent the means of triplicate samples expressed as % control.

Fig 5 shows that BW755C inhibited the response to PMA, 10^{-7}mol/L, in a dose-dependent manner with ID_{50} of 22 µg per mL. This curve is very similar to that in Fig 2, which shows the effect of BW755C on GCT-CM–induced myelopoiesis. Similar to the results shown in Fig 3, NDGA inhibited the response to PMA at concentrations greater than 5×10^{-5}mol/L.

Erythropoiesis

BFU-E were counted after 14 days of culture using an inverted microscope. In the absence of added erythropoietin, no BFU-E grew. In the presence of 2 units of EPO per mL, there were 177 ± 50 BFU-E colonies per 10^6 MDC plated.

Effects of LPO inhibitors on EPO response. BW755C inhibited the respone of blood MDC to EPO in a dose-dependent manner with ID_{50} of 5 µg per mL. Bone marrow WMNC were similarly inhibited by BW755C (see Table 4). BH completely inhibited blood MDC-derived BFU-E with ID_{50} of 15 µg per mL, while NDGA and 1P3P were only partially inhibitory.

DISCUSSION

The main findings of our studies are that drugs known to inhibit both LPO and CO enzymes block in vitro CSF-induced myelopoiesis, PMA-induced monocytopoiesis, and BPA/EPO-induced erythropoiesis. In contrast, indomethacin, at a concentration that inhibits only CO activity, has no

Table 3. CFU-GM Colony Growth After 24-Hour Preincubation With BW755C

	BW755C µg/mL 0	50	0 (50)
CFU-GM Colonies	59 ± 7.8	60 ± 8.2	0 ± 0
n	4	4	2

Blood MDC were incubated for 24 hours in liquid cultures, as described in Materials and Methods, with either 0 or 50 µg per mL of BW755C. The cells were then washed three times and plated in methycellulose with GCT-CM. Colonies were counted 14 days later. The third column, 0 (50), signifies that these cells were preincubated for 24 hours without BW755C, then plated for 14 days in the presence of GCT-CM and BW755C 50 µg per mL. The data shown are the means ± SEM of results from the indicated number of experiments (n).

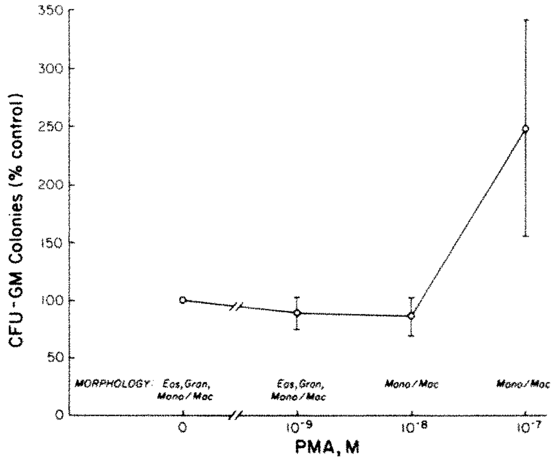

Fig 4. Response to PMA alone, without an exogenous source of CSF. PMA was added at the indicated concentrations on day 0 of 14-day cultures of blood MDC. Morphology of plucked colonies was determined by Wright-Giemsa and nonspecific esterase staining. Each point represents the mean ± SEM of the results from four experiments.

effect. These data suggest that certain LPO metabolites of AA are important mediators of myelopoiesis and erythropoiesis.

We have shown by two parameters that the LPO inhibitors are not toxic to the MDC population containing progenitor cells. First, cell viability was not adversely affected by BW755C or NDGA compared to control groups. Second, the inhibitory effect of these drugs was reversible after a 24-hour period of preincubation.

The differences in the dose-response curves for BW755C compared to NDGA (Figs 2 and 3) may reflect the different patterns of inhibition of the various lipoxygenase enzymes by these two drugs. Salari et al[28] have shown in a mixture of human leukocytes and platelets that NDGA at concentrations less than 5 × 10^{-6}mol/L stimulates production of 12-LPO products, and inhibits their production at higher concentrations. In contrast, NDGA blocks production of 5-LPO metabolites at all concentrations. BW755C inhibits both 5- and 12-LPO at all concentrations, though it is more potent against the 5-LPO than the 12-LPO.

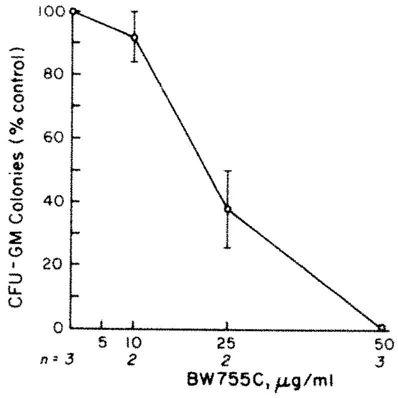

Fig 5. Inhibition of PMA by BW755C. PMA, 10^{-7}mol/L, was added along with BW755C on day 0 of 14-day cultures of blood MDC. n, number of experiments represented.

Table 4. Effect of BW755C on BFU-E Growth

BW755C μg/mL	BFU-E, % Control	
	Blood MDC	Bone Marrow WMNC
0	100	100
1	76	38
10	19	3
25	11	0
50	0	—

Blood MDC or bone marrow WMNC were plated for BFU-E colony growth, as described in Materials and Methods, in the presence of BW755C at the indicated concentrations. BFU-E were counted after 14 days. The results shown are the means of duplicate samples from two representative experiments.

We studied PMA-induced monocytopoiesis because PMA shares several biologic properties with CSF,[9] and because of the known effects of PMA on LPO metabolites in other systems. It has been shown, for example, to stimulate production of LPO metabolites in K562 cells and other tumor cell lines.[19] We found that the LPO inhibitors were just as effective against PMA-induced monocytopoiesis as they were against CSF-induced myelopoiesis. Moreover, the specific patterns of response of PMA to NDGA compared to BW755C were the same as those of CSF to these two inhibitors. These data suggest that LPO activation may be the final common intracellular pathway by which CSF, PMA, and BPA/EPO transmit their signals generated at the cell membrane.

The actual target cell for the LPO inhibitors in our system is not absolutely identified since the populations studied included cells other than progenitor cells. However, the effect was evident in cohorts depleted of monocytes and T cells and was not dependent upon endogenous production of CSF. We have no evidence for requirement of an accessory cell in the system.

We postulate that the effect of LPO inhibitors in blocking myelopoiesis and erythropoiesis is directly on the progenitor cells. In some models of cell proliferation mediated by growth factors or mitogens, it has been shown that certain key lipoxygenase metabolites are produced in response to a signal delivered by the growth factor at the level of the cell membrane.[5,10,11,33-35] Such a response may occur in progenitor cells, but the exact sequence of steps that link the ligand-receptor interaction to the intracellular metabolism of AA remains to be elucidated.

In summary, we have shown that inhibitors of LPO metabolism of AA block in vitro myelopoiesis and erythropoiesis. LPO metabolites may be critical intracellular messages for growth factors that stimulate hematopoietic progenitor cells to proliferate and differentiate.

REFERENCES

1. Burgess AW, Metcalf D: The nature and action of granulocyte-macrophage colony stimulating factors. Blood 56:947, 1980
2. Peschle C: Erythropoiesis. Ann Rev Med 31:303, 1980
3. Stanley ER, Guilbert LJ, Tushinski RJ, Bartelmez SH:

CSF-1—A mononuclear phagocyte lineage-specific hemopoietic growth factor. J Cell Biochem 21:151, 1983

4. Shadduck RK, Pigoli G, Caramatti C, Degliantoni G, Rizzoli V, Porcellini A, Waheed A, Shiffer L: Identification of hemopoietic cells responsive to colony-stimulating factory by autoradiography. Blood 62:1197, 1983

5. Burgess AW: Granulocyte-macrophage colony stimulating factor. Lymphokine Res 3:64, 1984 (abstr)

6. Stuart RK, Hamilton JA, Sensenbrenner LL, Moore MAS: Regulation of myelopoiesis in vitro: Partial replacement of colony-stimulating factors by tumor-promoting phorbol esters. Blood 57:1032, 1981

7. Abraham JL, Smiley R: Modification of normal human myelopoiesis by TPA. Blood 58:1119, 1981

8. Sieber F, Stuart RK, Spivak JL: Tumor-promoting phorbol esters stimulate myelopoiesis and suppress erythropoiesis in cultures of mouse bone marrow cells. Proc Natl Acad Sci 78:4402, 1981

9. Guilbert LJ, Nelson DJ, Hamilton JA, Williams N: The nature of TPA-stimulated hemopoiesis, colony stimulating factor requirement for colony formation, and the effect of TPA on [^{125}I] CSF-1 binding to macrophages. J Cell Physiol 115:276, 1983

10. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y: Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 257:7847, 1982

11. Gilmore T, Martin GS: Phorbol ester and diacylglycerol induce protein phosphorylation at tyrosine. Nature 306:487, 1983

12. Nathan DG, Sytkowski A: Editorial retrospective. Erythropoietin and the regulation of erythropoiesis. N Engl J Med 308:520, 1983

13. Hirata F, Schiffman E, Venkatasubramanian K, Salomon D, Axelrod J: A phospholipase A_2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. PNAS 77:2533, 1980

14. Gentile P, Byer D, Pelus LM: In vivo modulation of murine myelopoiesis following intravenous administration of prostaglandin E_2. Blood 62:1100, 1983

15. Hamilton JA: Glucocorticoids and prostaglandins inhibit the induction of macrophage DNA synthesis by macrophage growth factor and phorbol ester. J Cell Physiol 115:67, 1983

16. Rossi GB, Migliaccio AR, Migliaccio G, Letteri F, Dirosa M, Mastroberardino G, Peschle C: In vitro interactions of PGE and cAMP with murine and human erythroid precursors. Blood 56:74, 1980

17. Golde DW, Earsch M, Cline MJ: Potentiation of erythropoiesis in vitro by dexamethasone. J Clin Invest 57:57, 1976

18. Ohuchi K, Levine L: α-tocopherol inhibits TPA-stimulated deacylation of cellular lipids, prostaglandin production, and changes in cell morphology of Madin-Darby canine kidney cells. Biochim Biophys Acta 619:11, 1980

19. Valone FH, Obrist R, Tarlin N, Bast RC Jr: Enhanced arachidonic acid lipoxygenation by K562 cells stimulated with TPA. Cancer Res 43:197, 1983

20. Samuelsson B: Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. Science 20:568, 1983

21. Showell HJ, Naccache PH, Borgeat P, Picard S, Vallerand P, Becker EL, Sha'afi RI: Characterization of the secretory activity of LTB_4 toward rabbit neutrophils. J Immunol 128:811, 1982

22. Metz S, Van Rollins M, Strife R, Fujimoto W, Robertson RP: Lipoxygenase pathway in islet endocrine cells. J Clin Invest 71:1191, 1983

23. Snyder GD, Capdevila J, Chacos N, Manna S, Falck JR: Action of luteinizing hormone-releasing hormone: Involvement of novel arachidonic acid metabolites. PNAS 80:3504, 1983

24. Ilekis J, Benveniste R: JEG—3 cells: Influence of the arachidonic acid pathway on EGF and PMA stimulation of choriogonadotropin. Lymphokine Res 3:90, 1984 (abstr)

25. Kelly JP, Johnson MC, Parker CW: Effect of inhibitors of arachidonic acid metabolism on mitogenesis in human lymphocytes: Possible role of thromboxanes and products of the lipoxygenase pathway. J Immunol 122:1563, 1979

26. Bailey JM, Bryant RW, Low CE, Pupillo MB, Vanderhoek JY: Regulation of T-lymphocyte mitogenesis by the leukocyte product 15-HETE. Cell Immunol 67:112, 1982

27. Bailey JM, Bryant RW, Low CE, Pupillo MB, Vanderhoek JY: Role of lipoxygenases in regulation of PHA and phorbol ester-induced mitogenesis, in Samuelsson B, Paoletti R (eds): Leukotrienes and Other Lipoxygenase Products. New York, Raven Press, 1982, p 341

28. Seaman WE: Human natural killer cell activity is reversibly inhibited by antagonists of lipoxygenation. J Immunol 131:2953, 1983

29. Johnson HM, Torres BA: Leukotrienes: Positive signals for regulation of α-interferon production. J Immunol 132:413, 1984

30. Salari H, Braquet P, Borgeat P: Comparative effects of indomethacin, acetylenic acids, 15-HETE, nordihydroguaiaretic acid and BW755C on the metabolism of arachidonic acid in human leukocytes and platelets. Prost Leuk Med 13:53, 1984

31. Blackwell GJ, Flower RJ: 1-phenyl-3-pyrazolidone: An inhibitor of arachidonate oxidation in lung and platelets. Proceedings of the PBS, 29–30th March, 1978, p. 360P

32. Kaplan ME, Clark C: An improved rosetting assay for detection of human T lymphocytes. J Immunol Methods 5:131, 1974

33. Higgs GA, Flower FJ, Vane JR: A new approach to anti-inflammatory drugs. Biochem Pharm 28:1959, 1979

34. Myers RF, Siegel MI: Differential effects of anti-inflammatory drugs on lipoxygenase and cyclooxygenase activities of neutrophils. Biochem Biophys Res Commun 112:586, 1983

35. Hirata F: The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. J Biol Chem 256:7730, 1981

Effects of Therapy With T101 Ricin A-Chain Immunotoxin in Two Leukemia Patients

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Two leukemia patients, refractory to chemotherapy, were treated with T101-ricin A-chain immunotoxin (T101 IT). Patient 1 (T-ALL) received a single 13.5 mg dose of T101 IT IV (12-hour infusion). Patient 2 (B-CLL) was treated with a daily 25 mg dose of T101 IT IV (two-hour infusion) over three consecutive days. Patient 2 also received 300 mg of chloroquine IM on days two and three as enhancer. In vivo binding of T101 IT was demonstrated by FACS analysis using either an antimouse Ig-FITC or anti–A-chain-FITC antibodies. Following IT therapy, the expression of T65 antigen on target cells dropped to 50% and 20% of pretreatment levels, respectively. In patient 1, circulating blast cells remained unsaturated during therapy while in patient 2, cells were fully saturated for four to six hours following each infusion. Pharmacokinetic studies showed a rapid clearance of T101 IT after IV administration. Anti-mouse and anti-A-chain antibodies could not be detected. There were no treatment-related adverse effects. In patient 1 a rapid but transient decrease of target cells was observed, possibly related to the administration of the antibody part of T101 IT. In contrast, patient 2 showed a 40% reduction of the lymphocyte count, which remained stable over a period of 2 weeks. Such a clinical benefit following IT therapy in patient 2 could be ascribed to the absence of circulating free antigen and the complete saturation of target cells.

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LARGE scale production of murine monoclonal antibodies (MoAb) reacting with human malignant cells offers new potential for antitumor serotherapy. In recent years, a number of clinical trials using monoclonal antibodies have been reported for the treatment of various malignant diseases, including non-B non-T acute lymphoblastic leukemias (ALL),[1] T-ALL,[2] B- and T-cell lymphomas,[3-7] chronic lymphocytic leukemias (CLL),[8-10] or solid tumors.[11-13] These studies showed, in most cases, a clinical benefit consisting mainly of a rapid but transient decrease of circulating malignant cells in leukemia patients. In some cases of lymphoma or metastatic carcinoma, a tumor regression was found.[7,13] One of the possible limiting factors could be an insufficient cytotoxic effect of murine MoAbs, which needs a mediation via the natural host effector cells.

Antibody carriers of toxins (immunotoxins) offer a promising alternative, since immunotoxins have been found to induce high specific cell killing efficacy without the help of complement or cellular immune mechanism in a number of both in vitro and in vivo animal models.

Different toxins have been used, including ricin or its A-chain subunit[14-16] as well as diphtheria toxin,[17] gelonine,[18] or Pokeweed Antiviral Protein.[19] Ricin or ricin A-chain immunotoxins have already been used in clinical hematology for bone marrow purging in both autologous and allogeneic bone marrow transplantation.[20,21]

Some free nonspecific binding sites of whole ricin immunotoxins account for their ability to bind nonspecifically to any cell. In contrast, ricin A-chain immunotoxins hardly possess any nonspecific binding sites and thus they present a high potential for in vivo use, despite their lower efficacy due to the absence of the B chain and its helper function. The in vivo efficacy of ricin A-chain immunotoxins has been evaluated in virus-induced T2 mouse leukemia models and results are promising.[22]

We have prepared an immunotoxin, coupling A chain of ricin with T101 monoclonal antibody[23] which reacts with a 65 KD glycoprotein (T65) expressed on the cell membrane of mature T cells, of most T-cell–derived lymphoid neoplasias, B-CLL,[23] and a minority of B-cell non-Hodgkin lymphomas.[24,25] T101 does not react with other hematopoietic cells and has not been detected on nonhematopoietic tissue.[23]

Animal toxicology studies were conducted in rats, mice, and monkeys.[26] Following a single IV injection, the LD10 in mice was about 10 mg/kg. The LD50 was about 23 mg/kg with lesions observed in the liver and kidney. In 16 macaques receiving T101-IT, tolerance was good at 1 or 3 mg/kg, two out of four animals died at doses of 9 mg/kg five and eight days later with gastrointestinal ulcerations and liver and renal necrosis. No toxicity was observed following five daily IV injections of 0.02 to 2 mg/kg.

Supported by the toxicologic safety of T101-IT in animals, we have started preliminary clinical trials to evaluate tolerance and efficacy of T101 IT. This article reports the results of the treatment by T101-IT of two patients suffering from a malignant blood disease refractory to chemotherapy (T-ALL and B-CLL, respectively).

CASE REPORTS

Patient no. 1. E.B, a 23-year-old woman, was referred for treatment of ALL to the department of Hematology, Toulouse. Complete remission was induced with vincristine, cyclophosphamide, and prednisone in June 1981. CNS prophylaxis was obtained by intrathecal methotrexate and cranial irradiation. The patient was subsequently treated with 6-mercapto-purine and methotrexate until May 1983, when a relapse occurred. Despite intensive chemotherapy, which included vincristine, rubidazone, L-asparaginase, and prednisone, a second remission was not achieved. Chemotherapy was discontinued in July 1983. E.B. was readmitted into hospital in September 1983. Physical examination revealed widespread ecchymoses, fever, and hepatosplenomegaly. Laboratory studies showed a white blood cell count of 20,700 per μL with 99% lymphoblasts. Hematocrit was 33%, and the platelet count 11,000 per μL. Renal and hepatic function tests were normal. Informed consent was

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obtained as for protocol on file with Toulouse University Medical Center Institutional Review Board (Protocol PCL 243–83), and therapy with T101 IT was started.

Patient no. 2. J.I., a 57-year-old man, was admitted to University Hospital Henri-Mondor in August 1983, with axillary lymphadenopathy and hepatosplenomegaly. White cell count was 36,600 per μL. 95% of the cells were small mature lymphocytes. A marrow aspirate showed dense lymphocytic infiltration (85%) and the absence of immature forms, and the diagnosis of CLL was made. No treatment was given. One month later, the patient underwent splenectomy for splenic hematoma. Subsequently, cyclosphamide (150 mg daily) was administered, but discontinued after 7 months because no improvement was obtained. A 3-month course of chemotherapy was started with cyclophosphamide, velbe, and prednisone (CVP) with no clinical benefit. Both CHOP regimen and high-dose chlorambucil therapy (18 mg daily) failed to improve the hematologic status. Chemotherapy was definitely discontinued in October 1984. At that time, the patient presented with hepatomegaly, diffuse lymphadenopathy, hyperleucocytosis (475,000 per μL), anemia, and thrombocytopenia (95,000 per μL). WBC remained stable between January and March 1985 varying between 350,000 and 420,000 per μL. In March 1985, the tumoral status was unchanged. Hemoglobin was 10.2 g/100 mL; WBC count was 367,000 with 99% mature small lymphocytes. Renal function was normal. Liver function was slightly impaired with a mild elevation of SGOT, SGPT, and of alkaline phosphatases (59,112 and 231 IU, respectively). The patient was considered unresponsive to standard chemotherapy. Informed consent and CHU Henri Mondor Human Protection Committee approval were obtained (Protocol PCL 537–84). Therapy with T101 IT was initiated.

MATERIALS AND METHODS

Immunotoxin preparation. Purified T101 monoclonal antibody (MoAb) was purchased from Hybritech Inc (La Jolla, Calif). Ricin A chain was disulphide linked to the T101 MoAb as described previously.[27] For in vivo use, T101 ricin A chain IT solution was tested for sterility (CFR 610–12 requirement), pyrogens (CFR 610–13 requirement), general safety (CFR 610–11 requirement), nucleid acid contamination, and presence of murine virus (LCM, polyoma, reovirus type 1 and 3, and MLV). Furthermore, mouse antibody production test was performed for FDA required type of viruses (Federal Register vol 49 no. 110, 1984).

Immunologic characterization of malignant cells. Peripheral blood samples were obtained from patients on heparinized syringes. Mononuclear cells were separated on Ficoll-Hypaque gradient.

Indirect immunofluorescence analysis was performed by flow cytometry with a FACS IV (Becton Dickinson, Mountain View, Calif). A panel of MoAbs was used including T101, 3A1 (Hybritech Inc), OKT3, OKT4, OKT8, OKT6 (purchased from Ortho Diagnostic Systems), IOT5 (anti-CALLA provided by Immunotech, France), 2G5 (monomorphic anti-class II molecule produced in our laboratory), B1, B2, B4 (Coultronics), and SAFI B3 (anti-B associated antigen produced in our laboratory).

For the identification of surface immunoglobulins, anti-IgM, anti-IgG, and anti-IgD MoAbs were purchased from Dakopatts (Santa Barbara, Calif); anti-IgA, anti-K, and anti-λ chain MoAbs from Yeda (Rehovot, Israel). MoAbs were used at the final concentration of 10 μg per mL. Fluorescein iso-thiocyanate (FITC) conjugated sheep F(ab')2 antimouse IgG secondary antibody was purchased from New England Nuclear (Boston, Mass) and used at a final concentration of 40 μg per mL for 45 minutes.

Quantification of MoAb binding was performed according to the method described by Poncelet.[28] Briefly, for each experiment, an internal standard curve was established by indirect immunofluorescence from different CEM subclones whose T101 binding levels (1,000; 5,000; 20,000; and 44,000 molecules per cell, respectively) had previously been determined by saturation experiments using radiolabeled T101. Using the standard curve, the mean fluorescence intensity of the T101-target cell reaction allowed to calculate the number of binding sites on the cell membrane.

Administration of T101 ricin A-chain immunotoxin. Two different treatment schedules were used in this study: Patient no. 1 received 13.5 mg of T101 ricin A chain as two six-hour infusions; patient no. 2 was treated with 25 mg of T101 IT daily as a two-hour infusion during three consecutive days (total dose: 75 mg). Each dose of T101 IT was diluted in 500 mL isotonic saline. Allopurinol was administered systematically. No prophylactic antihistamine drug was given. A 300 mg dose of Chloroquine was administered IM to patient no. 2 on days 2 and 3 as an enhancer agent.

Clinical study parameters. Toxicity: vital signs were monitored every 30 minutes during infusion. Electrolytes and renal and hepatic functions were assessed before, during, and after treatment, and once a week thereafter.

Clinical effect: peripheral WBC counts, hematocrit, and platelet counts were monitored each hour during the infusion and 4, 12, and 24 hours after each infusion. WBC counts were then monitored daily after treatment. Serial measurements of lymph nodes, liver, and spleen size were carried out daily.

Expression of T65 antigen. Blood samples were collected from patient no. 1 at 2, 4, 6, 8, and 12 hours on drug and 12 hours later, and from patient no. 2 at 0.5, 1, 2, 6, 12, and 24 hours after each administration.

Mononuclear cells were isolated from 2 mL heparinized venous blood samples by Ficoll-Hypaque gradient density centrifugation. 2.10**7 cells per mL were incubated with T101 MoAb at a final concentration of 10 μg per mL during one hour at 4 °C in order to saturate any free T65 antigen not previously bound in vivo to T101 IT. Cells were then washed three times and incubated with sheep F(ab')2 antimouse Ig-FITC as described above. FITC-labeled cells were washed once and fixed with 1% paraformaldehyde.[29] Cells were then examined using FACS analysis. The mean fluorescence intensity corresponded to the expression of the sum of free and T101-IT in vivo bound T65 antigen.[8]

In vivo binding of T101 IT measurement. Peripheral blood mononuclear cells were incubated directly with either antimouse Ig-FITC which binds to the antibody moiety of the T101 IT or goat antiricin A-chain-FITC which binds to the toxin moiety of the molecule.

Pharmacokinetic study. Evaluation of plasma level of T101 IT was performed using two different methods: immunoradiometric assay (IRMA-IT) and biological activity assay (BAA). IRMA-IT was performed as follows[30]: purified goat antiricin A-chain antibody (Ab1) was absorbed onto 96 studs of microtitration plates (Nunc-transferable solid phase (TSP) screening system) by incubating the studs overnight with Ab1 in PBS solution (10 μg per mL). The immunoabsorbent was then saturated with calf serum for two hours. Various dilutions of plasma samples in normal human plasma or known concentrations of IT solutions in normal human plasma were then added. The standard curve was then established. After two hours of incubation, the immunoabsorbent was washed with PBS. The detection of IT was performed by incubating the immunoabsorbent for two hours with radiolabeled 125-I purified goat antimouse IgG antibody in PBS containing 2% of calf serum. After washing out with PBS, the radioactivity was measured in a CG-30 gamma spectrometer (Intertechnique, France). The nonspecifically bound radioactivity, determined either by omitting Ab1 or by using normal human plasma, was subtracted. Each point represents the mean of three tests. Plasma concentration of T101 IT was calculated from standard curves.

BAA was performed as follows: CEM cells were resuspended at a concentration of 8.10 × 5 cells per mL in culture medium containing 100 nmol/L monensin. Fifty μL aliquots were placed into wells of microtiter plates. Fifty μL of various concentrations of IT or of serial dilutions of plasma samples in culture medium were then added and left to incubate with the CEM cells for 16 to 18 hours at 37 °C. Protein synthesis was measured by 14C-leucine incorporation into cell proteins.[14] Each point represents the mean of four tests. Results are expressed as the percentage of 14C-leucine incorporation relative to untreated control cells. The concentration of active T101 IT in plasma was established using the following formula:

Plasma IT concentration = IT IC50 × plasma DI50 where IT IC50 is the concentration of IT expressed in ricin A-chain content causing a 50% inhibition of 14C-leucine uptake; DI50 is the dilution of plasma causing a 50% inhibition of 14C-leucine uptake.

Antimouse Ig and anti–A-chain antibody production. The evaluation of host immune antimouse Ig and anti–A-chain antibody production was measured using the following immunoradiometric inhibition assay: for antimouse Ig evaluation, purified T101 MoAb was absorbed on 96 microtiter plates (Nunc-TSP-screening system) by incubating the studs overnight with T101 antibody at a concentration of 4.5 μg per mL in PBS. The immunoabsorbent was then saturated with calf serum for two hours at room temperature. Plasma samples (150 μL) were then added at various dilutions, and incubated for one hour at room temperature. The immunoabsorbent was washed once with PBS, then allowed to react with 125-I antimouse Ig antibody (TAGO). Radioactivity was measured in a gamma spectrometer. Plasma concentrations of antimouse Ig were calculated from standard curves established from experiments using various dilutions of antimouse Ig at known concentrations in normal human plasma. For anti–A-chain antibody production, purified ricin A chain was absorbed on 96 microtiter plates (Nunc-TSP-screening system) by incubating the studs for three hours at room temperature in PBS solution (800 ng per mL) and then saturated with FCS for three hours. Various dilutions of plasma samples were incubated for one hour at room temperature. The immunoabsorbent was washed once with PBS, then 125-I purified goat anti–A-chain antibody was added. Plasma concentrations of anti-A-chain Ig were evaluated from standard curves established from experiments using various dilutions of known concentrations of purified goat anti–A-chain in normal human plasma. Both these assays were found to detect antibody concentrations higher than 2 μg per mL.

Serum levels of chloroquine. Determination of chloroquine and its desethyl metabolite was performed with a liquid chromatographic method using fluorometric detection as reported by Alvan.[31]

RESULTS

Immunologic characterization of malignant cells. Prior to therapy, multiple analysis of the first patient's mononuclear cells showed that her circulating blast cells were T101+, 3A1+, DR-, SIg-, CALLA- (T-ALL cells). OKT11 and OKT4 were weakly positive while OKT8 was negative. Quantification of T101 binding showed a mean density of 14,000 T1 (T101) molecules per cell. Lymphocytes of the second patients were characterized SIg+ (IgMK), T101+, DR+, B1+, B4+, Ba1+, SAFI-B3+, B2−, PCA1−, PC1−, OKT3− (B-CLL cells). Cytofluorometric quantitative study evaluated the mean density of T1 molecules (T101) at 10,000 per cell.

Clinical effect. In patient no. 1, before therapy, blast cell count was 20,700 μL. It dropped to 50% of the pretreatment level in four hours, remaining stable during the following six hours and returning to initial level six hours after treatment (Fig 1). During the next three days, a rebound phenomenon was noted. On the fourth day, blast cell count was 30,000 per μL increasing progressively to 110,000 per μL on day 22 when the patient died from *E coli* septicemia (see Fig 2). In patient no. 2, before therapy, WBC count was 367,000 per μL (99% lymphocytes). A slight reduction of leucocytosis (20% to 25%) was found immediately after the first two administrations of T101 IT (Fig 3) followed each time by a rebound. Four days after the completion of therapy, WBC count was 230,000, remaining stable over 2 weeks. The patient died on day 19 of hemorrhage related to thrombocytopenia refractory to platelet transfusions (Fig 4). Neither patient showed a reduction in the size of lymph nodes, spleen, or liver or an improvement in platelet count or hemoglobin level.

Toxicity. In both patients, tolerance was excellent. No anaphylactoid reaction, hypotension, dyspnoea, chills, or malaise were ever observed. There was no increase in LDH or uric acid following therapy. There was no change in the estimated creatinine clearance following treatment. Patient no. 2 who had, before therapy, an abnormal SGOT (59 IU) level showed a slight but transient increase of SGOT level up to 112. SGPT and alkaline phosphatases serum levels remained stable.

Expression of T65 antigen. Expression of T65 antigen was evaluated by adding, in vitro, excess T101 antibody to cells obtained from T101 IT-treated patients. Thus, the mean intensity of fluorescence corresponded to the total T65 antigen bound both in vivo and in vitro. The expression of T65 antigen began to drop immediately after the beginning of infusion reaching 50% of the pretreatment level at the twelfth hour in patient no. 1 and 20% at the sixth hour in patient no. 2. After treatment, T65 antigen expression came back to pretreatment value in patient no. 1 while in patient no. 2 it remained at 50% of its initial level.

Binding of T101 IT and saturation of target cells. In order to study the binding of T101 IT, cells obtained from the treated patients were allowed to react either with an antimouse Ig-FITC or with anti–A-chain-FITC antibody. Indirect immunofluorescence assays confirmed the binding of T101 IT to the target cells in vivo. In both patients, the two curves were very similar (results not shown). Saturation of circulating target cells was determined by comparing these curves with the curve of T65 antigen expression as described above. In patient no. 1, circulating blast cells remained unsaturated during the time of therapy (see Fig 5). In patient no. 2, during the first two hours, cells remained unsaturated, probably due to the time needed for IT to bind to target cells. During the following four hours, target cells became saturated since no free T65 antigen could be detected, as shown in Fig 6. The same picture was observed during the following infusions.

Pharmacokinetic studies. T101 IT levels were evaluated by both immunoradiometric assay (IRMA-IT) and biologic activity assay (BAA). In patient no. 1, IT level remained stable during the 12-hour infusion (see Fig 7). IT level IRMA estimation gave a concentration of IT within a range of 115 to 450 ng/mL. BAA showed much lower IT concentrations, within the range of 4 to 8 ng/mL. After the

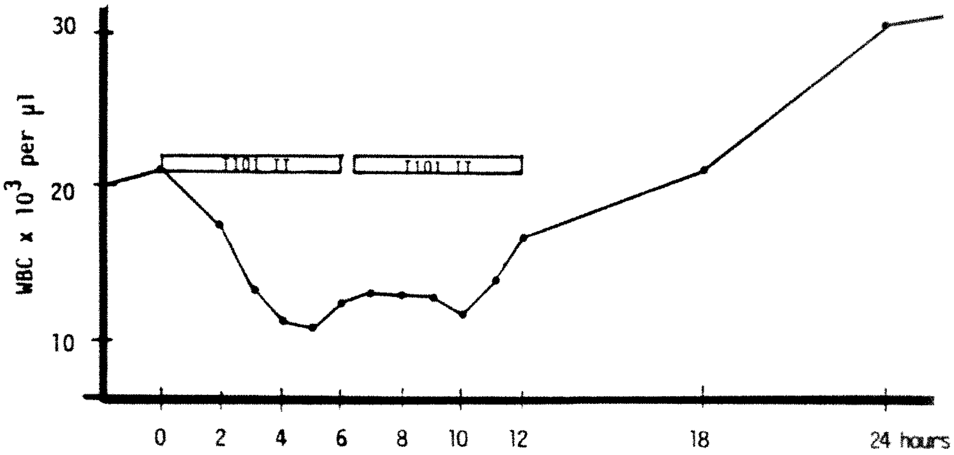

Fig 1. Patient no. 1: WBC levels during and following T101 IT therapy (hours).

completion of IT therapy, IRMA showed a rapid elimination of IT with a concentration of approximately 2.5 ng/mL, 24 hours after the end of the infusion. No BAA activity could be found after IT therapy, as shown in Fig 7. In patient no. 2, both IRMA and BAA showed an initial plateau concentration of 600 ng/mL during the first two hours, corresponding to the infusion of IT. As in patient no. 1, this plateau is followed by a rapid decrease of IT level (see Fig 8). However, in contrast with patient no. 1, IRMA and BAA gave similar results during and after therapy.

Host immune response. No significant levels of either antimouse or anti-A-chain Ig were detected in patient no. 2, 2 weeks after the end of treatment. These tests could not be performed in patient no. 1.

Dosage of chloroquine. HPLC analysis of chloroquine serum levels performed on days 2 and 3 in patient no. 2 showed a peak at 30 minutes at concentrations of 270 ng/mL (day 2) or 320 ng/mL (day 3; approximately 10** – 6 mol/L). The clearance of chloroquine was rapid since six hours after the IM administration, serum levels were evaluated at 50 and 90 ng/mL on days 2 and 3, respectively.

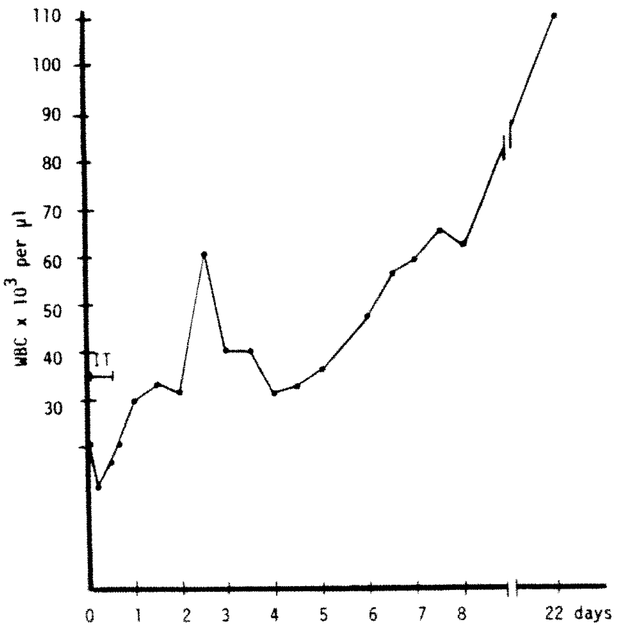

Fig 2. Patient no. 1: WBC levels during and following T101 IT therapy (days).

DISCUSSION

Two patients with end-stage malignant blood diseases in refractory phase were treated with T101 ricin A-chain immunotoxin (T101 IT). Different regimens of administration were used in terms of dose and time of infusion. No treatment-related complications occurred following IT therapy. Furthermore, the in vivo biologic effects were promising, including immunotoxin binding to target cells. In one patient (patient no. 2) a sustained reduction of circulating tumor cells was observed.

Clinical effect of IT therapy was evaluated by leukemia cell counts. In both patients, a rapid and transient reduction of circulating leukemia cells was observed immediately after the initiation of therapy. From both clinical experience and in vitro background, a body of evidence suggests that the rapid removal of target cells under IT therapy is probably due to the administration of the antibody part of the T101 IT, rather than to a biologic effect of the immunotoxin. In fact, such an effect has been currently reported after therapy of leukemia patients with MoAbs alone, including T101 or leu 1 MoAbs.[7-10] Moreover, from experimental in vitro

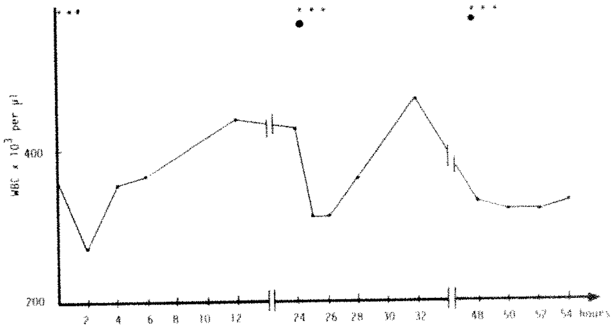

Fig 3. Patient no. 2: WBC levels during T101 IT and chloroquine therapy (hours). (***) 25 mg T101 IT as a two-hour infusion on days 1, 2, 3. (●) 300 mg chloroquine IM on days 2, 3.

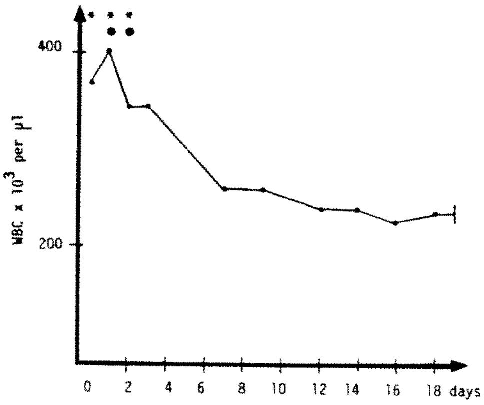

Fig 4. Patient no. 2: WBC levels during and following T101 IT therapy (days). (***) 25 mg T101 IT as a two-hour infusion on days 1, 2, 3. (●) 300 mg chloroquine IM on days 2, 3.

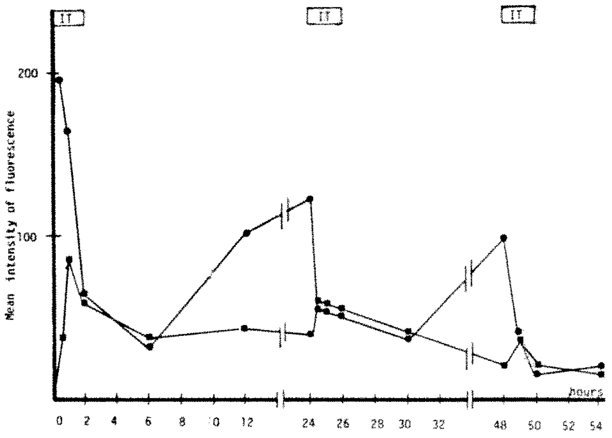

Fig 6. Patient no. 2: Expression of T65 antigen (●——●) and binding of T101 IT (■——■) during and following T101 IT therapy. After each infusion of 25 mg IT, cells were saturated during four to six hours.

findings, we can assume that after treatment with T101 IT, a much longer time period is needed to induce the destruction of target cells. For example, a 60-hour period is needed to obtain 90% inhibition of protein synthesis in vitro from T101 IT-treated CEM cells in the absence of NH4CL or monensin as activating agents.[14] Furthermore, cells that were unable to proliferate could remain intact for a long time before they died. The delay for effective cell destruction was found to be 7 to 10 times longer than that required for protein synthesis inhibition.[14] Therefore, an "immunotoxin response" can only be expected several days after T101 IT therapy. Patient no. 1 did not show such a response. However, the fast growth kintetics of ALL cells in this patient, as suggested by the rapid posttreatment increase of blast cells, could have deleted any biologic effect of T101 IT. More interesting results were found in patient no. 2 since WBC counts dropped by 40% within five days and remained stable over 2 weeks before the patient died on day 19 from a hemorrhagic syndrome. As an alternative hypothesis to a T101 IT effect, such a decrease of WBC count could be explained by the natural evolution of the disease, although the WBC had remained very stable over 3 months before IT therapy. Another explanation could be the possible effect of the antibody part of IT. However, a number of considerations can be put forward against this interpretation: (1) the five-day delay of the response was poorly consistent with an "antibody effect" as extensively described by other authors[1,7–9]; (2) a durable response was found to be extremely unusual after MoAb therapy since, in the most favorable cases, the decrease of circulating cells in leukemia patients

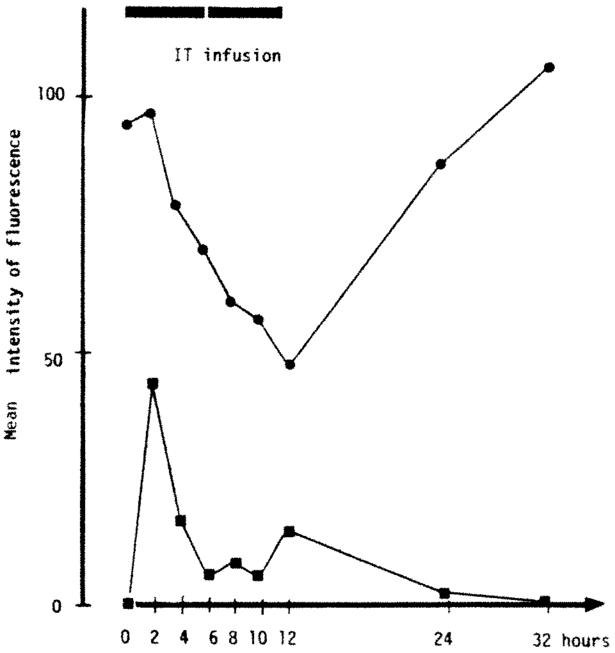

Fig 5. Patient no. 1: Expression of T65 antigen (●——●) and binding of T101 IT (■——■) during and following T101 IT therapy. The difference between the two curves reflects the amount of unsaturated cells. Saturation index never exceeded 30%.

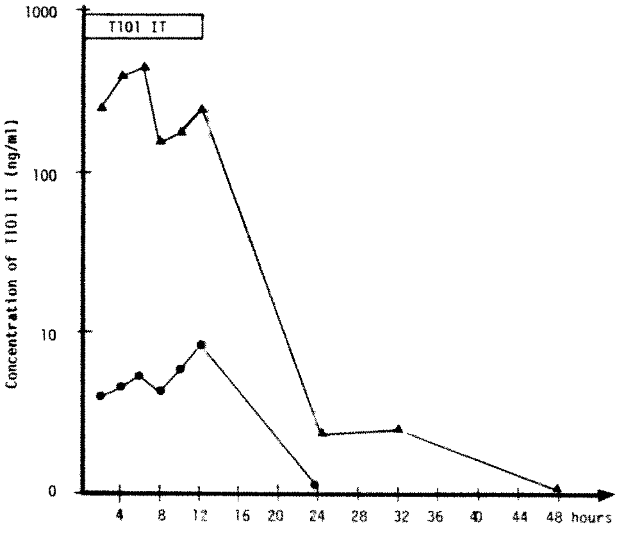

Fig 7. Patient no. 1: Pharmacokinetic study: T101 IT levels were evaluated during and following therapy using immunoradiometric assay (IRMA-IT) (▲——▲) or biologic activity assay (BAA) (●——●).

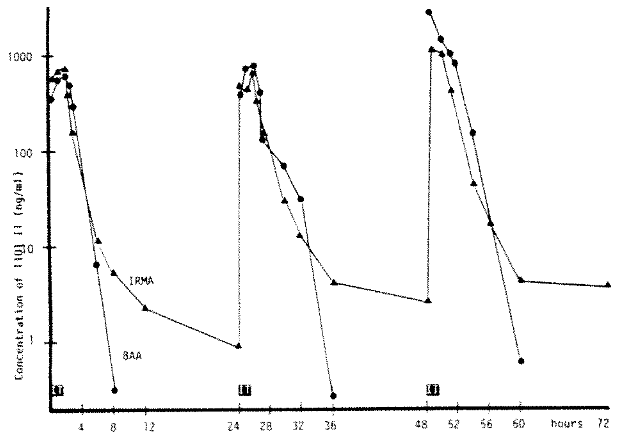

Fig 8. Patient no. 2: Pharmacokinetic study: T101 serum levels were evaluated during and following therapy using immunoradiometric assay (IRMA-IT) (▲——▲) or biologic activity assay (BAA) (●——●).

did not last more than four to seven days[9,10]; (3) a cytoreduction of 40% of malignant cells was consistent with experimental findings, since we have found that T101 IT, at a concentration of 10** − 8 M of A chain, could induce in vitro, in the absence of activator, a cytoreduction of 0.5 to 1 log on CEM cells after 4 to 24 hours incubation, as assessed by the cloning assay.[32] These findings suggested also that chloroquine administered to patient no. 2 as activating agent did not enhance in vivo T101 IT cell killing efficacy. In fact, chloroquine serum level concentration was found to be lower than 10** − 6 mol/L while a concentration of 10** − 4 mol/L was needed to be active in vitro.[33] These results suggested that chloroquine might not be a potential IT activating agent in clinical practice.

Tolerance was excellent in both patients, respectively, treated with 13.5 mg and 75 mg total dose of IT. No clinical side effects were observed nor liver or kidney toxicity found. The rate of administration of T101 MoAb was low in patient no. 1 (0.0125 mg/mn) but much higher in patient no. 2 (0.14 mg/mn). Previous studies indicated that at an infusion rate of over .7 mg/mn, toxic side effects occurred.[9]

Another objective of this study was to demonstrate in vivo the binding of T101 IT to tumor cells. In both patients, we demonstrated the localization of T101 IT on circulating leukemic cells, and we evaluated the expression of T65 antigen and the saturation index of target cells. In both patients, the expression of total T65 antigen (bound and unbound to IT) decreased rapidly from the onset of treatment. This phenomenon has been extensively discussed elsewhere[7,9,10] and might be the result of the additive effect of two different mechanisms: (1) an immunoselection of the target cells expressing a high density of T65 antigen which are rapidly removed by the reticulo-endothelial system; (2) T65 antigen modulation since this mechanism was found to occur both in vitro[34] and in vivo.[35] In contrast to MoAb therapy, surface antigen modulation may represent a favorable phenomenon for IT therapy. Indeed, Schroff et al have demonstrated that the modulation of T65 antigen is consistent with an internalization of the molecule.[35] Thus, this mechanism may enhance incorporation of the conjugate into target cells.

Nonsaturation of target cells was found to represent a possible limiting factor of IT therapy as shown in patient no. 1 in whom the mean fluorescence intensity of malignant cells stained with either antimouse Ig or anti–A-chain antibodies represented less than 50% of the mean fluorescence intensity corresponding to T65 antigen. The protocol of administration was therefore modified and a larger dose of IT administered. In patient 2, we found that target cells were entirely saturated for four to six hours following each administration of 25 mg of IT. Complete saturation of target cells in patient 2 could explain the 40% cytoreduction of circulating malignant cells in agreement with in vitro efficacy.

The reappearance of T65 antigen after IT therapy represented another phenomenon. In both patients, full or partial T65 antigen reexpression was found on the cell membrane of target cells within 12 hours after treatment. As suggested by Dillman et al,[10] this phenomenon might be the result of two different mechanisms: either the release of nontargeted cells from lymph nodes and bone marrow, as shown by 51 Cr-labeling studies, or the intrinsic reexpression of T65 molecules on the cell membrane of target cells observed in vitro.[34] Whatever the mechanism, the reexpression of T65 antigen suggested the potential benefit of daily repeated infusions.

Another aim of this study was to confirm in humans, the pharmacokinetic data obtained in animals, which showed a rapid clearance of T101 IT, evidently distinct from that observed with T101 MoAb alone.[30] Two different methods were used: immunoradiometric (IRMA) and biologic activity (BAA) assays. In patient no. 1, both IRMA and BAA showed a plateau of activity, followed by a rapid decrease of IT serum levels. Nevertheless these two assays gave a clearly distinct estimation of IT serum plateau level during IT therapy, ranging from 115 to 450 ng/mL (ie, approximately 0.3 to 1.3 10** − 8 mol/L of A chain) for IRMA and from 4 to 8 ng/mL for BAA. This difference suggests the presence, in the serum of patient 1, of circulating free T65 antigen which could have blocked the antibody binding site of T101 IT and thus interfered with BAA estimation. In patient 2, IRMA and BAA confirmed the rapid clearance of IT serum levels with similar estimation of IT serum plateau level, corresponding to a concentration of 600 ng/mL (ie, 2.10** − 8 mol/L of A chain). This finding suggests the absence of blocking factor in the serum of the B-CLL patient. In this regard, it has to be pointed out that Dillman et al did not find circulating T65 antigen in their series of CLL patients.[10]

Another major problem encountered in MoAb therapy is the production of endogenous antimouse antibodies in patients. In patient no. 2 neither human antimouse Ig nor human anti–A-chain Ig in the serum were found 2 weeks after IT therapy. Unhappily, the patient died soon after, and our data need to be confirmed by studies in a large group of B-CLL patients. However, this raises the question of multiple treatment with ricin A-chain ITs.

Finally, this study showed that ricin A-chain ITs represent a safe and promising alternative to serotherapy with MoAbs alone. High dose of IT administration was found to improve the saturation of target cells while repeated injections take advantage of the reexpression of the target antigen following IT therapy. T101 IT is being currently tested in a series of B-CLL patients to determine the toxicity associated with infusion of escalating doses and to evaluate the clinical feasibility of repeated injections. Furthermore, Ricin A-chain ITs may, in the future, become more potent in vivo in the presence of activator agents such as free[37] or bound B chains[38] or in the presence of drugs with potentiating properties such as carboxylic ionophores or, at a lesser degree, lysosomotropic amines.[33] Furthermore, a better understanding of the pharmacokinetics of ricin A-chain IT could lead to a considerable improvement of their in vivo potential.

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REFERENCES

1. Ritz J, Pesando JM, Sallan SE, Clavell LA, Notis-McConarty J, Rosenthal P, Schlossman SF: Serotherapy of acute lymphoblastic leukemia with monoclonal antibody. Blood 58:141, 1981

2. Bernard A, Gosse C, Pico J, Favre E, Raynal B, Hayat M, Lemerle J, Charbord P, Boumsell L: Chute de taux des lymphoblastes circulants par administration intraveineuse de l'anticorps monoclonal A50 chez un malade ayant une leucémie aiguë lymphoblastique à cellules T. Nouv Presse Med 11:3173, 1982

3. Nadler LM, Stashenko P, Hardy R, Kaplan WD, Button LN, Kufe DW, Antman KH, Schlossman SF: Serotherapy of a patient with a monoclonal antibody directed against a human lymphoma-associated antigen. Cancer Res. 40:3147, 1980

4. Miller RA, Maloney DG, Warnke R, Levy R: Treatment of B cell lymphoma with monoclonal antiidiotype antibody. N Engl J Med 306:517, 1982

5. Miller RA, Levy R: Response of cutaneaous T cell lymphoma to therapy with hybridoma monoclonal antibody. Lancet 2:226, 1981

6. Linch DC, Beverly PCL, Newland A, Turnbull A: Treatment of a low grade T cell proliferation with monoclonal antibody. Clin Exp Immunol 51:133, 1983.

7. Miller RA, Oseroff AR, Stratte PT, Levy R: Monoclonal antibody therapeutic trials in seven patients with T-cell lymphoma. Blood 62:988, 1983

8. Dillman RO, Shawler DL, Sobol RE, Collins HA, Beauregard JC, Wormsley SB, Royston I: Murine monoclonal antibody therapy in two patients with chronic lymphocytic leukemia. Blood 59:1036, 1982

9. Foon KA, Schroff RW, Bunn PA, Mayer D, Abrams PG, Fer M, Ochs J, Bottino GC, Sherwin SA, Carlo DJ, Herberman RB, Oldham RK: Effects of monoclonal antibody therapy in patients with chronic lymphocytic leukemia. Blood 64:1085, 1984

10. Dillman RO, Shawler DL, Dillman JB, Royston I: Therapy of chronic lymphocytic leukemia and cutaneous T-cell lymphoma with T101 monoclonal antibody. J Clin Oncol 2:881, 1984

11. Shen JW, Atkinson B, Koprowski H, Sears H: Binding of murine immunoglobulin to human tissues after immunotherapy with anticolorectal carcinoma monoclonal antibody. Int J Cancer 33:465, 1984

12. Schroff RW, Woodhouse CS, Foon KA, Oldham RK, Farrell M, Klein RA, Morgan AC: Intratumor localization of monoclonal antibody in patients wtih melanoma treated with antibody to a 250,000-Dalton melanoma-associated antigen. JNCI 74:229, 1985

13. Houghton AN, Mintzer D, Gordon-Caro C, Welt S, Fliegel B, Vadhan S, Carswell E, Melamed MR, Oettgen HF, Old LJ: Mouse monoclonal IgG3 antibody detecting GD3 ganglioside: A phase I trial in patients with malignant melanoma. Proc Natl Acad Sci USA 82:1242, 1985

14. Jansen FK, Blythman HE, Carriere D, Casellas P, Gros O, Gros P, Laurent JC, Paolucci F, Pau B, Poncelet P, Richer G, Vidal H, Voisin GA: Immunotoxin: Hybrid molecules combining high specificity and potent cytotoxicity. Immunol Rev 62:185, 1982

15. Thorpe PE, Ross WCJ: The preparation and cytotoxic properties of antibody-toxin conjugates. Immunol Rev 62:119, 1982

16. Vitetta ES, Krolick KA, Uhr JW: Neoplastic B-cells as targets for antibody-ricin A chain ITs. Immunol Rev 62:159, 1982

17. Thorpe PE, Ross NCJ, Cumber AJ, Hinson CA, Edwards DC, Davies AJS: Toxicity of diphtheria toxin for lymphoblastoid cells is increased by conjugation to antilymphocytic globulin. Nature 271:752, 1978

18. Stirpe F, Olsnes S, Pihl A: Gelonin, a new inhibitor of protein synthesis, non toxic to intact cells. Isolation, characterization and a preparation of cytotoxic complexes with concanavalin. Am J Biol Chem 255:6947, 1980

19. Ramakrishnan S, Houston LL: Prevention of growth of leukemia cells in mice by monoclonal antibodies directed against the 1.1 antigen disulfide linked to two ribosomal inhibitors: Pokeweed antiviral protein or ricin A chain. Cancer Res 44:1398, 1984

20. Maraninchi D, Novakovitch G, Laurent G, Mascret B, Tubiana N, Derocq JM, Casellas P, Gastaut JA, Perrimond H, Carcassone Y: Bone marrow transplantation after in vitro purging with T101-ricin A-chain immunotoxins: A phase I clinical study, in Dicke KA, Spitzer G, Zander AR (eds): Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. University of Texas, MD Anderson Hospital and Tumor Institute at Houston, 1985, p 425

21. Filipovich AH, Vallera DA, Youle RJ, Stong RC, Neville DM, Kersey JH: Marrow purging with immunotoxins in allogeneic and autologous bone marrow transplantation: Pilot clinical studies, in Dicke KA, Spitzer G, Zander AR (eds): Autologous Bone Marrow Transplantation. Proceedings of the First International Symposium. University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, 1985, p 403

22. Blythman HE, Bord A, Buisson I, Jansen FK, Richer G, Thurneyssen O: Antitumor effect of immunotoxins in mouse and human tumor models, in Peeters H (ed): Protides of the Biological Fluids. Oxford, Pergamon Press, 1984, p 421

23. Royston I, Majda JA, Baird SM, Meserve BL, Griffiths JC: Human T cell antigens defined by monoclonal antibodies: The 65,000 dalton antigen of T cells (T65) is also found on chronic lymphocytic leukaemia cells bearing surface immunoglobulin. J Immunol 125:725, 1980

24. Burns BF, Warnke RA, Doggett RS, Rouse RV: Expression of a T cell antigen (Leu 1) by B cell lymphomas. Am J Pathol 113:165, 1983

25. Al Saati T, Laurent G, Caveriviere P, Rigal F, Delsol G: Reactivity of Leu 1 and T101 monoclonal antibodies with B cell lymphomas (correlations with other immunological markers). Clin Exp Immunol 58:631, 1984

26. Remandet B, Gouy D, Vic P, Mazue G: Toxicological evalua-

tion of an immunotoxin in rats and monkeys by intravenous route (abstr). Int J Immunopharmac 7:402, 1985

27. Gros O, Gros P, Jansen FK, Vidal H: Biochemical aspects of immunotoxin preparation. J Immunol Methods 81:283, 1985

28. Poncelet P, Carayon P: Cytofluorometric quantification of cell-surface antigens by indirect immunofluorescence using monoclonal antibodies. J Immunol Methods 85:65, 1985

29. Lanier LL, Warner NL: Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J Imm Methods 47:25, 1981

30. Bourrie B, Casellas P, Blythman H, Jansen FK: Study of the plasma clearance of antibody ricin A chain immunotoxins: Evidence for specific recognition sites on the A chain that mediate rapid clearance of the immunotoxin. Eur J Biochem 1985 (in press)

31. Alvan G, Ekman L, Lindstrom B: Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography. J Chromatogr 229:241, 1982

32. Casellas P, Canat X, Fauser AA, Gros O, Laurent G, Poncelet P, Jansen FK: Optimal elimination of leukemic T cells from human bone marrow with T101-ricin A-chain immunotoxin. Blood 65:289, 1985

33. Casellas P, Bourrie B, Gros P, Jansen FK: Kinetics of cytotoxicity induced by immunotoxins. Enhancement by lysosomotropic amines and carboxyl ionophores. J Biol Chem 259:9359, 1984

34. Shawler DL, Miceli MC, Wormsley SB, Royston I, Dillman RO: Induction of in vitro and in vivo antigenic modulation by the anti human T-cell monoclonal antibody T101. Cancer Research 44:5921, 1984

35. Schroff RW, Farell MM, Klein RA, Oldham RK, Foon KA: T65 antigen modulation in a phase I monoclonal antibody trial with chronic lymphocytic leukemia patients. J Immunol 133:1641, 1984

36. Schroff FW, Foon KA, Beatty SM, Oldham RK, Morgan AC: Human antimurine immunoglobulin responses in patients receiving monoclonal antibody therapy. Cancer Research 45:879, 1985

37. Youle RJ, Neville DM: Kinetics of proteinsynthesis inactivation by ricin-anti thy 1.1 monoclonal antibody hybrids. Role of the ricin B subunit demonstrated by reconstitution. J Biol Chem 257:1598, 1982

38. Vitetta ES, Cushley W, Uhr JW: Synergy of ricin A chain-containing immunotoxins and ricin B chain-containing immunotoxins in in vitro killing of neoplastic human B cells. Proc Natl Acad Sci USA 80:6332, 1983

Cytogenetic Features and Serum Lactic Dehydrogenase Level Predict a Poor Treatment Outcome for Children with Pre-B–Cell Leukemia

By Ching-Hon Pui, Dorothy L. Williams, David K. Kalwinsky, A. Thomas Look, Susan L. Melvin, Richard K. Dodge, Gaston Rivera, Sharon B. Murphy, and Gary V. Dahl

Leukemic cells from 89 (24%) of 369 children with newly diagnosed acute lymphoblastic leukemia (ALL) were found to have a pre-B immunophenotype. By comparison with blasts having the common ALL phenotype, the pre-B cells were more likely to have a DNA index <1.16 ($P = 0.02$), a pseudodiploid karyotype ($P < 0.001$), and a chromosomal translocation ($P = 0.001$). Increased serum lactic dehydrogenase levels ($P = 0.001$) were also characteristic of pre-B ALL; otherwise, the clinical and laboratory features of the two groups were similar. A nonrandom chromosomal translocation, t(1;19)(q23;p13.3), was identified in blast cells from 16 (23%) of the 70 patients with pre-B ALL and adequate chromosome banding studies; different translocations were found in 11 of the remaining patients. The presence of any chromosomal translocation in the pre-B group was significantly related to a higher leukocyte count, an increased level of serum lactic dehydrogenase, an increased percentage of S-phase cells, black race, and a blast cell DNA index < 1.16. Four presenting features were found to confer an increased risk of treatment failure among pre-B patients: pseudodiploidy, chromosomal translocation, black race, and higher serum lactic dehydrogenase level. In a multivariate analysis, pseudodiploidy emerged as the strongest factor for predicting relapse in pre-B ALL. The frequent association of chromosomal abnormalities of known adverse prognostic significance and high serum lactic dehydrogenase levels with pre-B–cell ALL explains, at least in part, the poor treatment outcome reported for children with this subtype of leukemia.

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PRE-B cells, or B-lymphocyte precursors that contain cytoplasmic heavy-chain immunoglobulin (CIg) but lack surface Ig, have been recognized in both animals[1-3] and human beings.[4] Following their original description of the pre-B immunophenotype in 4 of 22 cases of acute lymphoblastic leukemia (ALL) in 1978, Vogler et al[5,6] studied an additional 191 cases but were unable to show differences in presenting features or treatment outcome between patients with blasts of the pre-B phenotype and those with other forms of non-T, non-B ALL. More recently, Crist et al[7,8] reanalyzed the results of therapy for the same group of children and 171 additional patients after extended follow-up and concluded that the pre-B–cell phenotype is a high-risk feature with independent prognostic significance. However, the biologic basis for the poor prognosis apparently conferred by this marker remains uncertain. We report here that a majority of cases of pre-B ALL (87%) are characterized by one or more high-risk prognostic features: a blast cell DNA index <1.16,[9] a pseudodiploid karyotype,[10,11] a chromosomal translocation,[11-13] and an increased serum lactic dehydrogenase level (LDH).[14] These findings help to clarify the poor prognosis that has been attributed to the pre-B immunophenotype.

MATERIALS AND METHODS

From February 1980 to January 1985, 461 patients with newly diagnosed ALL were admitted consecutively to St Jude Children's Research Hospital. During the same time period, eight other children were diagnosed as having B-cell leukemia (surface Ig$^+$) and were not entered in the study. Three hundred sixty-nine patients (80%) had complete blast cell immunophenotyping, including CIg testing. These 218 boys and 151 girls, ranging in age from 1 month to 18 years (median, 4.9 years), were admitted to the study with informed written consent.

All patients were subjects of two Total Therapy studies that incorporated different treatment protocols based on the patients' initial risk features and subsequent randomization.[15,16] The lack of a uniform treatment and the relatively small number of pre-B–cell patients in each treatment arm precluded comparison of event-free survival times among different immunophenotypic groups.

Lymphoblast immunophenotype. Leukemic blasts from bone marrow aspirates were separated on a Ficoll-Hypaque gradient, and only samples containing >85% blasts were assayed. They were considered positive for heat-stable E-rosette formation at 37 °C if at least 2% of the blasts formed rosettes. Cell-surface antigens were detected by a standard indirect immunofluorescence assay with monoclonal antibodies, including J5 (common ALL antigen, CALLA),[17] HLA-DR, T101,[18] T-11,[19] and T-3.[20] Two investigators independently counted 200 cells in each immunofluorescence assay; the results were judged to be positive if 40% or more of the blasts had surface fluorescence. Cell-surface Ig was identified by direct fluorescence with fluoresceinated goat antihuman immunoglobulin. CIg was identified by a direct method that employed fluoresceinated F(ab')$_2$ goat antihuman μ-chains (Southern Biotechnology, Birmingham, Ala)[5]; positive cases were those in which 10% or more blasts contained CIg. Depending on their pattern of reactivity, the samples were classified as having a common (CALLA$^+$, HLA-DR$^+$, E$^-$, T$^-$, CIg$^-$, SIg$^-$), T (T$^+$, E-rosette$^+$, HLA-DR$^-$), pre-B (CIg$^+$, HLA-DR$^+$, CALLA$^{\pm}$), or undifferentiated (HLA-DR$^+$) immunophenotype. Blast cells were also classified according to French-American-British (FAB) criteria by two independent observers; any discrepancies between the L1 and L2 classifications were resolved by the scoring system of Bennett et al.[21]

DNA content determinations. Leukemic marrow samples were stained with the DNA-specific dye propidium iodide. Histograms of DNA content were analyzed for the percentage of S-phase cells; the DNA index (DI, or the ratio of DNA content in leukemic G_0/G_1 cells versus normal diploid G_0/G_1 cells) was determined by flow cytometry as previously described.[9] DI correlates closely with chromosome number; hence, leukemic cells with a normal chromosome complement have a DI of 1.0.

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Cytogenetic studies. Bone marrow samples were prepared with a direct technique developed for ALL and a modified trypsin-Giemsa technique for chromosome banding.[22] After study of at least 10 metaphases (mean 20) and preparation of karyotypes, cases were classified as either hyperdiploid (>50 chromosomes), pseudodiploid (46 chromosomes with numerical or structural alterations, or both), or "other" (normal karyotype, hypodiploid, or chromosome complement in the 47 to 49 range).[10] The pseudodiploid group also included cases with multiple stem lines containing a pseudodiploid component. A clone was considered abnormal if it met criteria of the Second International Workshop on Chromosomes in Leukemia.[23]

Statistical analysis. Distributions of clinical and biologic features among patients with pre-B–cell versus common ALL were compared by the Pearson chi-square test. The presenting features in cases of pre-B ALL, with or without a chromosomal translocation, were compared by the chi-square test or Mann-Whitney test. Treatment results were analyzed only for the subset of pre-B patients with adequate follow-up.[15] Time-to-failure curves were constructed by the Kaplan-Meier procedure,[24] with differences analyzed by the Cox-Mantel test.[25] Time to failure was defined as the interval between achievement of remission and relapse or death due to any cause. Patients who did not enter complete remission were assigned a failure time of zero. The influence of potentially significant prognostic factors on time to failure was estimated with the Cox proportional-hazards model,[26] which permits comparison of treatment outcome for two or more subsets of patients while simultaneously adjusting for the effect of other factors (covariates) in the model. The variable tested included age, sex, race, leukocyte count, hemoglobin level, platelet count, serum LDH level, liver size, spleen size, FAB classification, CNS leukemia, percentage of S-phase cells, DI, karyotype ploidy, and chromosomal translocations.

RESULTS

The presenting features of patients with either the pre-B (n = 89) or common (n = 203) form of ALL are summarized in Table 1. Lymphoblasts from 82 (92%) of the former group expressed CALLA but none had receptors for sheep erythrocytes or T-cell surface antigens. Cytoplasmic immunoglobulin was detected in 12% to 100% (median, 76%) of the blast cells. Clinical and laboratory findings in the two groups were similar, except that patients with pre-B ALL were more likely to have blasts with a DI < 1.16, a pseudodiploid karyotype and a chromosomal translocation, as well as a higher serum LDH level. In fact, 87% of the pre-B cases had one or more of the above high-risk features.

Table 1. Differences Among Presenting Features of Patients With Pre-B and Common ALL*

		Pre-B	Common	*P* Value
Lactic dehydrogenase level (U/L)	<300	15	53	0.001
	300–1000	37	73	
	>1000	27	15	
DNA index	≥1.16	11	49	0.02
	<1.16	74	145	
Karyotype ploidy	Hyperdiploid	13	72	<0.001
	Pseudodiploid	38	45	
	Other	19	63	
Chromosomal translocation	Absent	43	113	0.001
	Present	27	25	

*All other comparisons yielded similar results.

Chromosome banding studies, performed successfully for 70 children with pre-B–cell ALL, revealed a nonrandom chromosomal translocation, t(1;19)(q23;p13.3), in the blast cells of 16 patients (23%) and an array of different chromosomal translocations in 11 others: t(2;3)(p14;p14), t(2;15)(q33;q15), t(4;11)(q21;q25), t(4;14;)(q25;q32), t(5;9)(q11;p21), t(7;9)(cen;cen), t(7;9)(q22;p21), t(8;12)(p21;q13), t(9;11)(p13;q25), t(9;12)(p22;p12), and t(9;22)(q34;q11). Thus, blast cells from 27 (39%) of 70 patients had a chromosomal translocation. Hyperdiploidy (>50 chromosomes) was noted in 13 patients, none of whom had a translocation. Seven patients had normal karyotypes. The remaining 23 children had other chromosomal abnormalities.

The presenting features of patients with pre-B ALL characterized by the 1;19 translocation did not differ from those of patients having other translocations. However, analysis of clinical and laboratory findings according to the presence or absence of any chromosomal translocation indicated that this abnormality was significantly more likely to

Table 2. Comparison of Presenting Features Associated with Pre-B–Cell ALL, With or Without a Chromosomal Translocation

Features	With Translocation (*n* = 27)	Without Translocation (*n* = 43)	*P* Value
Age (median years)	4.1	3.9	NS
Sex (% boys)	59	51	NS
Race (% black)	26	2	0.003
Leukocyte count (median × 10^9/L)	28.5	11.5	0.02
Hemoglobin level (median × g/dL)	7.2	7.7	NS
Platelet count (median × 10^9/L)	53	38	NS
CNS leukemia (% present)	19	9	NS
Mediastinal mass (% present)	0	0	NS
Liver size (median in cm)*	3	3	NS
Spleen size (median in cm)*	3	2	NS
LDH level (median × U/L)	2060	404	0.002
FAB classification (% L1)	93	95	NS
% S-phase cells (% ≤6.8)	0	34	0.005
DNA index (% <1.16)	100	76	0.005
Pseudodiploidy (%)	81	35	<0.001

Abbreviations: CNS, central nervous system; LDH, lactic dehydrogenase; FAB, French-American-British; NS, not significant ($P > 0.05$).

*Palpable below the costal margins.

be associated with black race, a higher leukocyte count, an increased serum LDH level, an increased percentage of S-phase cells, and a pseudodiploid karyotype (Table 2).

When event-free survival times of the pre-B cases were analyzed with the Cox-Mantel test, several features were found to be associated with a poorer treatment outcome: pseudodiploidy, black race, higher serum LDH level, and chromosomal translocation (Fig 1). Patients with apparently random translocations were as likely to fail therapy as were those with the nonrandom t(1;19). Age, sex, leukocyte count, liver or spleen size, FAB subtype, hemoglobin level, platelet count, CNS leukemia, percentage of S-phase cells, and DNA index had no prognostic importance. By multivariate Cox regression analysis, the factor most predictive of time to failure was pseudodiploidy (data not shown).

DISCUSSION

This study provides insight into the poorer treatment outcome others have ascribed to the pre-B–cell immunophenotype. By comparison with common ALL, cases of pre-B leukemia were more likely to be associated with a higher serum LDH level, a blast cell DNA index <1.16, a pseudodiploid karyotype, and a chromosomal translocation—features with known adverse effects on treatment outcome.[9-14] This contrasts with observations in an earlier study,[27] in which the distribution of chromosomal abnormalities among children with pre-B ALL paralleled that of the non-T–cell group. We attribute this discrepancy to our improved technique for identifying cytogenetic abnormalities.[28] Most of the patients studied earlier did not have chromosome banding of sufficient quality to rule out pseudodiploidy or translocations. Indeed, the 54% frequency of pseudodiploidy we report for cases of pre-B ALL is much higher than the 15% previously published.[27]

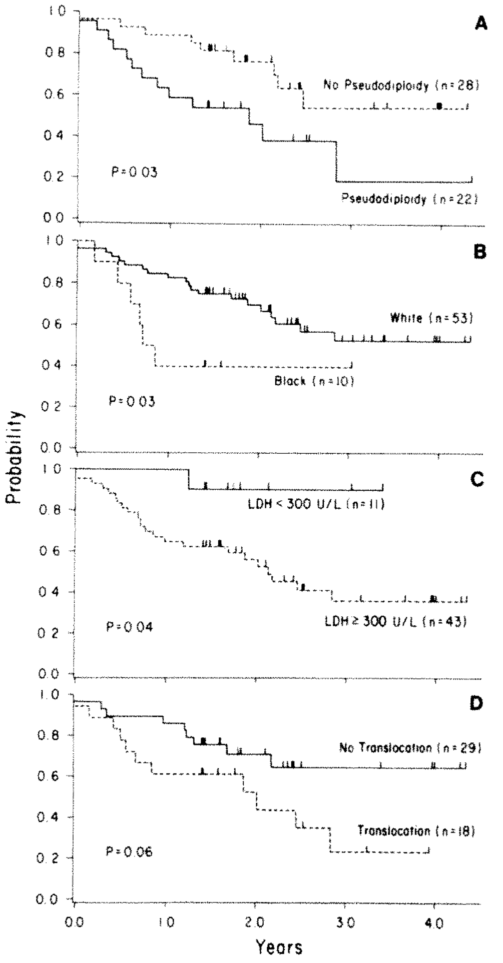

Fig 1. Comparison of time-to-failure rates among pre-B cases according to (A) karyotype ploidy, (B) race, (C) serum LDH level, and (D) chromosomal translocation.

Earlier cytogenetic studies of pre-B–cell leukemia consist largely of case reports, including Philadelphia chromosome (Ph)-positive chronic myelogenous leukemia in lymphoblastic crisis,[29-31] Ph-positive ALL,[32] and ALL with the 14q+ chromosomal abnormality,[33-35] a marker of Burkitt's lymphoma. However, subsequent study of 74 children with this immunologic subtype of ALL disclosed only one Ph-positive case and none with the 14q+ marker.[27] Similarly, there was only a single case of Ph-positive ALL in our series. We found, however, that 40% of children with pre-B ALL had blast cell chromosomal translocations. The t(1;19)(q23;p13.3), a specific chromosomal translocation previously associated with pre-B ALL,[36-38] was found in 60% of cases with translocations. It may be important that the gene encoding the insulin receptor was recently mapped to the distal short arm of chromosome 19 (bands p13.2 → p13.3), the site involved in the nonrandom translocation.[39] This finding suggests the intriguing possibility that the 1;19 rearrangement may alter the expression of the insulin receptor gene and hence contribute to malignant transformation or cell proliferation, although the basis for the apparent specificity of this alteration for the pre-B stage of differentiation remains unclear.

In general, patients with pre-B ALL and any chromosomal translocation were more likely to have high-risk features at diagnosis, including black race,[40,41] a high leukocyte count,[40] a high serum LDH level,[14] and a DNA index <1.16.[9] Carroll et al[37] have suggested that the 1;19 translocation may mark a subgroup of patients with pre-B-cell ALL who have a poor prognosis; however, Michael et al[38] reported that two of their four patients with this abnormality remained in remission for 17+ and 21+ months and suggested that the 1;19 translocation may not confer a poor prognosis. Our results support the idea that the t(1;19), as well as other translocations, is related to a poorer treatment outcome among patients with pre-B ALL. In fact, analysis of prognostic factors in childhood ALL has indicated that the presence of any translocation is associated with a sixfold greater risk of early treatment failure.[13]

Multivariate analysis indicated that pseudodiploidy was the high-risk factor with the greatest predictive strength; however, translocations and a DI <1.16 are closely related to this feature (Table 2),[9,10,13] so that the relative importance of the three variables may change with longer follow-up and accrual of additional patients. It may be important that serum LDH levels were approximately five times higher in cases with a translocation. We previously have shown that such increased levels are associated with a poor treatment outcome in childhood ALL and suggested that this measurement may provide a more reliable estimate of the total body burden of leukemic cells than does leukocyte count alone.[14] Patients with the highest LDH levels (>1,000 μ/L) were most likely to fail treatment, whereas those with the lowest levels (< 300 μ/L) had the lowest risk of failure.

Our findings also support the idea that the value of traditional clinical risk factors, such as leukocyte count and age, depends on immunophenotype. Further study is needed to define the relationship between immunophenotype and chromosomal abnormalities in predicting the treatment outcome of children with ALL, and more importantly, to select the optimal treatment program for each patient based on immunologic and genetic features of the leukemic blasts.

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REFERENCES

1. Osmond DG, Nossal GJV: Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. Cell Immunol 13:132, 1974

2. Ryser J-E, Vassalli P: Mouse bone marrow lymphocytes and their differentiation. J Immunol 113:719, 1974

3. Haywood AR, Simons M, Lawton AR, Cooper MD, Mage RG: Pre-B and B cells in rabbits: Ontogeny and allelic exclusion. Fed Proc 36:1295, 1977 (abstr)

4. Gathings WE, Lawton AR, Cooper MD: Immunofluorescent studies of the development of pre-B cells, B lymphocytes and imunoglobulin isotype diversity in humans. Eur J Immunol 7:804, 1977

5. Vogler LB, Crist WM, Bockman DE, Pearl ER, Lawton AR, Cooper MD: Pre-B-cell leukemia: A new phenotype of childhood lymphoblastic leukemia. N Engl J Med 298:872, 1978

6. Vogler LB, Crist WM, Sarrif AM, Pullen DJ, Bartolucci AA, Falletta JM, Dowell B, Humphrey GB, Blackstock R, van Eyes J, Metzgar RS, Cooper MD: An analysis of clinical and laboratory features of acute lymphocytic leukemias with emphasis on 35 children with pre-B leukemia. Blood 58:135, 1981

7. Crist W, Boyett J, Roper M, Pullen J, Metzgar R, van Eys J, Ragab A, Starling K, Vietti T, Cooper M: Pre-B cell leukemia responds poorly to treatment: A Pediatric Oncology Group Study. Blood 63:407, 1984

8. Crist WM, Grossi CE, Pullen DJ, Cooper MD: Immunologic markers in childhood acute lymphocytic leukemia. Semin Oncol 12:105, 1985

9. Look AT, Roberson PK, Williams DL, Rivera G, Bowman WP, Pui C-H, Ochs J, Abromowitch M, Kalwinsky D, Dahl GV, George S, Murphy SB: Prognostic importance of blast cell DNA content in childhood acute lymphoblastic leukemia. Blood 65:1079, 1985

10. Williams DL, Tsiatis A, Brodeur GM, Look AT, Melvin SL, Bowman WP, Kalwinsky DK, Rivera G, Dahl GV: Prognostic importance of chromosome number in 136 untreated children with acute lymphoblastic leukemia. Blood 60:864, 1982

11. Bloomfield CD, Lindquist LL, Arthur D, McKenna RW, LeBien TW, Nesbit MA, Peterson BA: Chromosomal abnormalities in acute lymphoblastic leukemia. Cancer Res 41:4838, 1981

12. Third International Workshop on Chromosomes in Leukemia 1980. Cancer Genet Cytogenet 4:96, 1981

13. Williams DL, Harber J, Murphy SB, Look AT, Kalwinsky DK, Rivera G, Melvin SL, Stass S, Dahl GV: Chromosomal translocations play a unique role in influencing prognosis in childhood acute lymphoblastic leukemia. Blood (in press)

14. Pui C-H, Dodge RK, Dahl GV, Rivera G, Look AT, Kalwinsky D, Bowman WP, Ochs J, Abromowitch M, Mirro J, Murphy SB: Serum lactic dehydrogenase level has prognostic value in childhood acute lymphoblastic leukemia. Blood 66:778, 1985

15. Pui C-H, Dahl GV, Bowman WP, Rao BN, Abromowitch M, Ochs J, Rivera G: Elective testicular biopsy during chemotherapy for childhood leukaemia is of no clinical value. Lancet ii:410, 1985

16. Rivera GK, Evans WE, Kalwinsky DK, Mirro J, Ochs J, Dow LW, Abromowitch M, Pui C-H, Dahl GV, Look AT, Crone M, Murphy SB: Unexpectedly severe toxicity from intensive early treatment of childhood lymphoblastic leukemia. J Clin Oncol 3:201, 1985

17. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF: A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 283:583, 1980

18. Royston I, Majda JA, Baird SM, Meserve BL, Griffiths JC: Human T-cell antigens defined by monoclonal antibodies: The 65,000-dalton antigen of T-cells (T56) is also found on chronic lymphocytic leukemia cells bearing surface imunoglobulin. J Immunol 125:725, 1980

19. Verbi W, Greaves MF, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G: Monoclonal antibodies OKT-11 and OKT-11A have pan-T reactivity and block sheep erythrocyte "receptors." Eur J Immunol 12:81, 1982

20. Reinherz EL, Kung PC, Goldstein G, Schlossman SF: Separation of functional subsets of human T cells by a monoclonal antibody. Proc Natl Acad Sci USA 76:4061, 1979

21. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: The morphological classification of acute lymphoblastic leukemia: concordance among observers and clinical correlations. Br J Haematol 47:553, 1981

22. Williams DL, Harris A, Williams KJ, Brosius MJ, Lemonds W: A direct bone marrow chromosome technique for acute lymphoblastic leukemia. Cancer Genet Cytogenet 13:239, 1984

23. Second International Workshop on Chromosomes in Leukemia 1979. Cancer Genet Cytogenet 2:89, 1980

24. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457, 1958

25. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163, 1966

26. Cox DR: Regression models and life tables. J R Stat Soc B34:187, 1972

27. Jackson JF, Pullen J, Williams D, Bowman P, Roper M, Carroll AJ, Vogler L, Crist WM, Starling KA, van Eys J: Cytogenetics of B-cell lineage acute lymphocytic leukaemias of childhood. Lancet ii:1334, 1982

28. Williams DL, Raimondi S, Rivera G, George S, Berard CW, Murphy SB: Presence of clonal chromosome abnormalities in virtually all cases of acute lymphoblastic leukemia. N Engl J Med 313:640, 1985 (letter)

29. Vogler LB, Crist WM, Vinson PC, Sarrif A, Brattain MG, Coleman MS: Philadelphia-chromosome-positive pre-B-cell leuke-

mia presenting as blast crisis of chronic myelogenous leukemia. Blood 54:1164, 1979

30. LeBien TW, Hozier J, Minowada J, Kersey JH: Origin of chronic myelocytic leukemia in a precursor of pre-B lymphocytes. N Engl J Med 301:144, 1979

31. Greaves MF, Verbi W, Reeves BR, Hoffbrand AV, Drysdale HC, Jones L, Sacker LS, Samaratunga I: "Pre-B" phenotypes in blast crisis of Ph' positive CML: Evidence for a pleuripotential stem cell "target." Leuk Res 3:181, 1979

32. Penchansky L, Whiteside T, Wollman M, Malatack J: Philadelphia chromosome-positive pre-B-lymphocytic leukemia in a child. Am J Pediatr Hematol Oncol 3:265, 1981

33. Kaneko Y, Rowley JD, Check I, Variakojis D, Moohr JW: The 14q+ chromosome in pre-B ALL. Blood 56:782, 1980

34. Imamura N, Kamada N, Kuramoto A: Pre-B–cell leukemia with the 14q+ chromosome. N Engl J Med 305:701, 1981 (letter)

35. Mufti GJ, Hamblin TJ, Oscier DG, Johnson S: Common ALL with pre-B–cell features showing (8;14) and (14;18) chromosome translocations. Blood 62:1142, 1983

36. Williams DL, Look AT, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S: New chromosomal translocations correlate with specific imunophenotypes of childhood acute lymphoblastic leukemia. Cell 36:101, 1984

37. Carroll AJ, Crist WM, Parmley RT, Roper M, Cooper MD, Finley WH: Pre-B cell leukemia associated with chromosome translocation 1;19. Blood 63:721, 1984

38. Michael PM, Levin MD, Garson OM: Translocation 1;19—a new cytogenetic abnormality in acute lymphocytic leukemia. Cancer Genet Cytogenet 12:333, 1984

39. Yang-Feng TL, Francke U: Gene for human insulin receptor: Localization to site on chromosome 19 involved in pre-B–cell leukemia. Science 228:728, 1985.

40. Kalwinsky DK, Roberson P, Dahl G, Harber J, Rivera G, Bowman WP, Pui C-H, Ochs J, Abromowitch M, Costlow ME, Melvin SL, Stass S, Williams DL, Murphy SB: Clinical relevance of lymphoblast biological features in children with acute lymphoblastic leukemia. J Clin Oncol 3:477, 1985

41. Kalwinsky DK, Rivera G, Dahl GV, Roberson P, George S, Murphy SB, Simone JV: Variation by race in presenting clinical and biologic features of childhood acute lymphoblastic leukaemia: Implications for treatment outcome. Leuk Res 9:817, 1985

Adverse Effect of Severe Marrow Fibrosis on Hematologic Recovery After Chemoradiotherapy and Allogeneic Bone Marrow Transplantation

By J. Rajantie, G.E. Sale, H.J. Deeg, D. Amos, F. Appelbaum, R. Storb, R.A. Clift, and C.D. Buckner

We reviewed the records and marrow biopsy specimens of 75 patients with leukemic, myelodysplastic, or myeloproliferative disorders to determine whether the presence of marrow fibrosis affected engraftment after allogeneic marrow transplantation. While 28 control patients without fibrosis achieved prompt engraftment, two of 32 patients (6%) with mild and five of 15 patients (33%) with severe fibrosis failed. The rate of myeloid recovery was significantly slower and the dependence on platelet and red blood cell transfusions was significantly longer in patients with severe fibrosis than in patients with no fibrosis. A finding of severe marrow fibrosis should therefore be taken into account when evaluating the risks and benefits of marrow transplantation.

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BONE MARROW transplantation is the treatment of choice for a variety of severe hematologic and immunologic disorders.[1,2] Although marrow rejection has been a problem in transfused patients with aplastic anemia if conditioned only with cyclophosphamide,[3] prompt and sustained engraftment has been the rule in patients transplanted with human leukocyte antigen (HLA)–identical marrow for neoplastic diseases following regimens including also total body irradiation.[4] Several case reports have suggested that the mere presence of fibrosis in pretransplant marrow does not necessarily jeopardize engraftment.[5-10] Even patients with acute (malignant) myelosclerosis have been successfully transplanted.[11,12] Since we observed some patients with marrow fibrosis in conjunction with various underlying diagnoses who failed to achieve prompt engraftment, we carried out a systematic retrospective analysis to determine the incidence of graft failure in this situation. A review of the records and marrow biopsy specimens of 75 patients revealed that patients with severe fibrosis are at greatly increased risk for delay or complete failure of sustained engraftment.

MATERIALS AND METHODS

Patients with marrow fibrosis. All patients transplanted at the Fred Hutchinson Cancer Research Center between February 1978 and November 1984 who were recorded as having fibrosis of any degree in their pretransplant marrow biopsies were gleaned from the pathology files. Biopsy slides were then rereviewed by two of us, and if adequate for evaluation, the amount of fibrosis was regraded (0–4) according to the criteria of Bauermeister.[13] In each biopsy, reticulin, collagen, or both were identified by special histologic stains (Wilder's silver stain or Gomori's trichrome stain, respectively). On the basis of fibrosis grade, those 47 patients who had adequate pretransplant marrow biopsy specimens were divided into two groups: severe fibrosis of grades 3 or 4 (15 patients) or mild fibrosis of grades 1 or 2 (32 patients).

Patients without marrow fibrosis. In addition, we collected data on a control group of 28 patients without detectable fibrosis at original review of slides (and confirmed during regrading). For this purpose we randomly selected for each patient with severe fibrosis two "case-controls" with the same or similar underlying diagnosis but without marrow fibrosis (for two patients only one control for each could be identified). Patients without fibrosis were matched with patients with severe fibrosis for age, sex, HLA barrier, and marrow cell dose. By definition the matching was not always possible (Table 1): controls for patients with "primary myelofibrosis" (acute or chronic myelofibrosis without other underlying diagnosis) came from the group of patients with chronic myeloid leukemia in either chronic or accelerated phase or in blast crisis depending on the percentage of blasts in the myelofibrosis patients' marrow. Data on four of the patients with acute (malignant) myelofibrosis[11] and three of the patients with preleukemia[14,15] have been published.

Transplantation procedure. Techniques for bone marrow transplantation used at our center have been described.[16] The preparative regimen always included two doses of cyclophosphamide (60 mg/kg) and total body irradiation at doses of 10 to 15.75 Gy (see Table 1). For prevention of GVHD, patients received either intermittent methotrexate (day 1 to 102),[1,17] daily cyclosporine (day −1 to 180),[18,19] or a combination of cyclosporine (given according to the same regimen) and methotrexate on days 1, 3, 6, and 11, only, as described in a canine model.[20] Five of the 75 patients were given marrow subjected in vitro to treatment with monoclonal antibodies and complement for T-cell depletion.[21] The diagnosis, grading, and treatment of acute GVHD has been discussed elsewhere.[1,22,23]

Evaluation of engraftment. For assessment of the relative rates of engraftment in the three groups (severe, mild, or no fibrosis) we compared the days after transplantion when total white blood cell (WBC) count reached 0.5, 1.0, or 2.0 × 10^9/L and when granulocyte counts reached 0.1, 0.5, 1.0, or 1.5 × 10^9/L. Similarly, the day when a patient first became platelet or red blood cell transfusioned independent was recorded. Total numbers of units transfusion independent was recorded. Total numbers of units transfused after transplantation in those intervals were counted.

Posttransplant evaluation of marrow fibrosis. Posttransplant marrow aspirates and biopsies were obtained on days 14, 21, 28, and 100, and at other times as clinically indicated. Presence or absence of fibrosis in these specimens was recorded.

Statistical analyses. In order to ensure disease homogeneity among compared groups, two patient populations were examined throughout the course of statistical analyses. First, only patients with the diagnosis of chronic myelogenous leukemia (CML) (N - 61) were included. The second analysis then incorporated all study patients (N - 75). Twelve outcome variables were examined: days posttransplant until reaching a given WBC (0.5, 1.0, 2.0 × 10^9/L) or granulocyte (0.1, 0.5, 1.0, 1.5 × 10^9/L) count, days until platelet or red blood cell (RBC) transfusion independence, days survived, days

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Table 1. Patient Characteristics

	Degree of Fibrosis		
Data	Severe*	Mild	None
No. of patients	15	32	28
Sex, M/F, (no. of patients)	9/6	18/14	19/9
Age (yr), (median and range)	26 (7-48)	30 (2-50)	30 (3-53)
Diagnosis (no. of patients)			
CML, CP	2	16	7
AP	2	3	13
BC	4	7	6
juvenile	0	1	0
ANLL, relapse	0	0	1
Preleukemia	2	1	1
Acute myelofibrosis	4	1	0
Chronic myelofibrosis	1	3	0
Dose of TBI (no. of patients)			
10 Gy	3	4	2
6 × 2 Gy	7	20	13
7 × 2.25 Gy	5	8	13
HLA incompat. donor (no. of patients)	4	0	4
Marrow dose ($\times 10^8$/kg) (median, range)	2.4 (0.3-7.4)	2.1 (0.3-4.9)	1.9 (0.3-8.9)
GVHD prophylaxis (no. of patients)†			
Cyclosporine (CSP)	2	14	11
Methotrexate (MTX)	10	13	16
CSP + MTX	3	5	1
T-cell depleted marrow	2	2	1

Abbrev: ANLL, acute nonlymphoblastic leukemia; CML, chronic myelogenous leukemia; AP, accelerated phase; BC, blast crisis; CP, chronic phase; TBI, total body irradiation.

*Severe, fibrosis grade 3 or 4; Mild, grade 1 or 2.

†See details in Materials and Methods.

to relapse, and days to relapse or death. Survival was determined as of March 31, 1985. In an attempt to detect possible differences in engraftment among patients with the three fibrosis levels (severe, mild, or none), a Mantel-Cox test was computed for each dependent variable.[24] Corresponding Kaplan-Meier curves were plotted.[25] To determine whether other factors, in addition to marrow fibrosis, affected engraftment and outcome, a proportional hazards model incorporating these variables (age, marrow cell dose, HLA identity or nonidentity, conditioning regimen, GVHD prophylaxis, and acute GVHD) was computed.[26] A step-wise technique was employed that allowed these six factors to enter and leave the model while the fibrosis variable remained in the model. Thus if any of the six additional factors explained a significant amount of the variability of any engraftment or survival variable, in addition to the variation accounted for by fibrosis differences, the factor was included in the final model. Three patients with severe and one with mild fibrosis died without ever having evidence of engraftment and therefore were considered not to be at risk for developing GVHD. These four patients were excluded from any analyses including GVHD as a variable. After statistically significant differences among the three fibrosis groups were found, pair-wise comparisons revealed which groups differed from the others. Corresponding P values were modified using Bonferroni's adjustment.[27]

RESULTS

Engraftment. Among the 15 patients with severe fibrosis, three died without evidence of sustained marrow engraftment (Table 2). Two additional patients had transient engraftment but because of subsequent graft failure required a second transplant. From the group of 32 patients with mild fibrosis, one never engrafted and died with marrow aplasia and one required a "booster" infusion of marrow. The difference in graft failure between patients with mild and severe fibrosis was statistically significant ($P = 0.046$ by Fisher's exact test). All 28 control patients without fibrosis had sustained engraftment. None of the patients with failure of engraftment had received marrow treated in vitro with anti-T-cell monoclonal antibodies and complement (see Materials and Methods).

Leukocyte reconstitution rates in peripheral blood, whether expressed as recovery of total WBC or granulocyte counts, did not differ between patients with mild and no fibrosis but was highly significantly delayed in patients with severe fibrosis (Fig 1). Similarly, the time to independence of platelet and RBC transfusions was significantly longer (Fig 2) and total numbers of units transfused were significantly larger for the group with severe fibrosis (Table 3).

Multivariate analyses showed that these results (rate of engraftment, transfusion independence) were not significantly affected by other factors such as patient age, marrow cell dose, underlying diagnosis, HLA matching, type of GVHD prophylaxis, presence of acute GVHD, and dose of total body irradiation (TBI; 10 v 6 × 2 v 7 × 2.25 Gy). In addition, the degree of fibrosis did not affect incidence or severity of GVHD.

Regression of marrow fibrosis. Follow-up marrow specimens were available in 44 of the 47 patients who had marrow fibrosis before transplantation. One to 11 (median

Table 2. Patients* Without Sustained Engraftment

UPN	Sex	Age (yr)	Diagnosis	TBI (Gy)	HLA†	Cell Dose (× 10^8/kg)	GVHD Prophyl	Evidence of Engraftment	Evidence of Rejection‡	Second Transplant§ (d)	Death
Patients with severe fibrosis											
1049	F	18	AMF	10	Id	2.4	MTX	No	—	No	d 22: septicemia
1866	M	26	CML, BC	7 × 2.25	Id	3.1	MTX	No	—	No	d33: aspergillosis
2602	F	7	preleuk	6 × 2	MM	2.8	CSP + MTX	No	—	d34 & 94	d113: intracerebral bleeding
1493	M	34	CML, AP	7 × 2.25	MM	1.0	MTX	Yes	Yes	d137	d664: bronchopneumonia
2208	F	26	AMF	6 × 2	Id	7.4	CSP	Yes	Yes	d69	d71:VOD, pneumonitis
Patients with mild fibrosis											
2096	F	24	CML, CP	6 × 2	Id	2.7	CSP + MT	No	—	No	d 21:VOD
814	M	27	CML, BC	6 × 2	Id	2.9	MTX	Yes	Yes	d118	d268: aspergillosis

Abbrev: AMF, acute myelofibrosis; CML, chronic myelogeneous leukemia; AP, accelerated phase; BC, blast crisis; CP, chronic phase; VOD, venocclusive disease.

*These patients are included in Figs 1, 2 and in Table 3.

†Id, genotypically identical; MM, two-antigen mismatch.

‡Loss of hemopoietic precursors in serial marrow biopsies. Return of host cells was identified by genetic markers only in UPN 2208.

§UPN 2208 and 2602 were reconditioned before later marrow infusion; UPN 814 and 1493 had a "booster" infusion without immunosuppressive pretreatment. They both then achieved a successful graft.

three) successful posttransplant biopsies were obtained per patient. Reversal of fibrosis was observed in 12 of 15 patients with severe and in 24 of 29 patients with mild fibrosis. Interestingly, fibrosis reappeared later in six of the former and in one of the latter group; this occurred concomitantly with graft rejection in three and leukemic relapse in four patients. We had histologic data on liver but not spleen at autopsy in only three of the patients with pretransplant fibrosis (Unique patient number, 814, 1866, and 2208). None had extramedullary hematopoiesis.

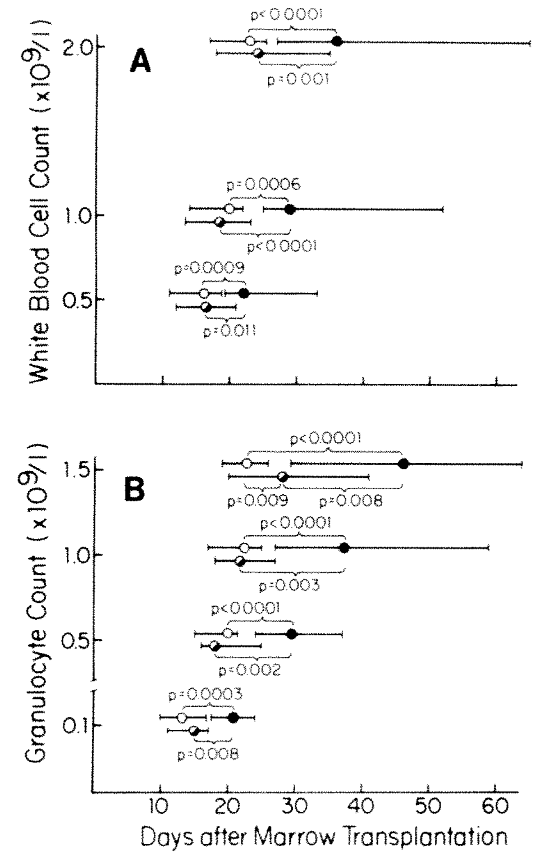

Fig 1. Days posttransplant at which the designated levels of white blood cell count (A) and granulocyte count (B) were reached. Medians and 25th to 75th percentile range are presented. *P* values have been computed from the respective Kaplan-Meyer plots (Mantel-Cox test). ○, no fibrosis; ◒, mild fibrosis; ●, severe fibrosis.

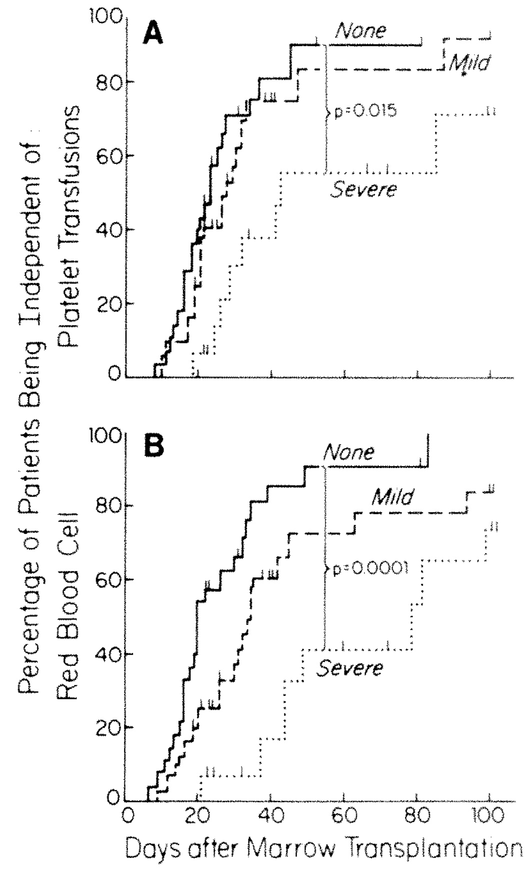

Fig 2. The cumulative probability of being platelet (A) or red blood cell (B) transfusion independent. Tic marks indicate patients who have been censored because of death before becoming transfusion independent. Only *P* values <0.05 are presented (Mantel-Cox test).

Table 3. Total Number* of Units of Red Blood Cells and Platelets Transfused After Bone Marrow Transplantation

Degree of Fibrosis	Number of Transfusions	
	Platelets	Red Blood Cells
Severe	220, 100–434	31, 15–40
Mild	88, 42–362 $P < 0.021$†	9, 7–23 $P < 0.006$†
None	44, 30–164	8, 6–20

*Median, 25th and 75th percentile.

†Only the difference between patients with severe and with no fibrosis was statistically significant.

Survival. Survival did not differ significantly among the three groups (Fig 3), whether computed as days survived, days to relapse, or days to relapse or death.

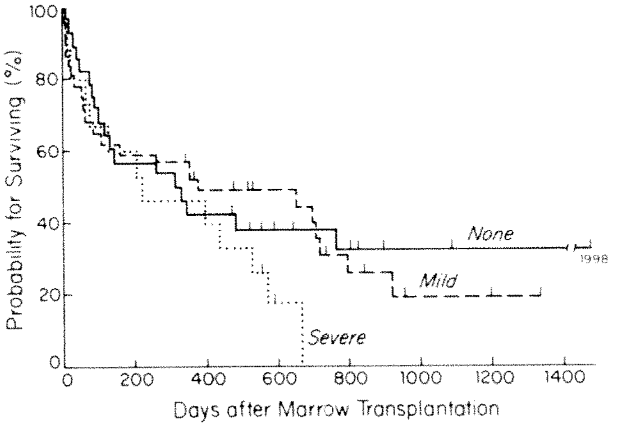

Fig 3. The cumulative probability for survival. Tic marks indicate patients still alive. The difference between the curves was not statistically significant (Mantel-Cox test).

DISCUSSION

Patients conditioned with TBI usually have sustained engraftment after HLA-identical marrow infusion.[1-2] A generally accepted concept is that space must be present in the marrow for transplanted cells to find their "niche" and to replicate. Therefore, there have been concerns that marrow fibrosis at the time of marrow transplantation might adversely affect engraftment, and transplantation for such patients was, initially, undertaken only reluctantly.[5] Surprisingly, however, several patients have been reported to have had a successful transplant despite fibrotic marrow.[5-12] These case reports, however, do not address whether marrow fibrosis might in some patients be accompanied by impaired hematologic recovery.

In the present study, we have carried out a systematic analysis of our experience over the last 7 years in an attempt to determine the influence of marrow fibrosis on engraftment. Although a majority of the patients achieved sustained engraftment, our results indicate that both mild and severe marrow fibrosis can adversely affect posttransplant hematopoietic reconstitution. However, this effect was consistent and highly significant only with severe grades of fibrosis. The effect of fibrosis was independent of other parameters such as marrow cell dose, type of GVHD prophylaxis, or HLA mismatching. It is worth noting, however, that among four patients given HLA nonidentical marrow two failed to achieve sustained engraftment (Table 2). This latter finding is in agreement with the observation in marrow transplants for other indications that the risk of failure of engraftment is higher with HLA nonidentical grafts.[28]

It is at present not clear why marrow fibrosis might have an adverse effect on engraftment. Marrow obliterated by dense fibrosis may be unable to provide an adequate microenvironment for normal hematopoietic reconstitution after myeloablative therapy, possibly by interfering with the delivery of vital nutrients, growth factors, or cellular mechanisms necessary for adequate stem cell proliferation,[29] or it is possible that persisting marrow fibrosis is simply a sign that the malignant process has not been ablated and it may be the malignant process itself that inhibits engraftment.

The situation is further complicated by the fact that the etiology of fibrosis itself is incompletely understood. Excessive production of fibrosis in the bone marrow is thought to be a secondary process. Marrow fibrosis is seen in association with a variety of neoplastic, myelodysplastic, and myeloproliferative disorders although it may arise idiopathically.[30-35] Chromosomal[30] and biochemical[31] studies indicate that marrow fibroblasts differ from the cell clone involved in leukemia proliferation. The fibrotic process can be reversed by intensive chemotherapy combined with or without TBI.[6-12,32] In the present study, regression of fibrosis was associated with successful engraftment and reappearance of fibrosis occurred concomitantly with either graft rejection or leukemic relapse. Although serial biopsies after bone marrow transplantation showed a relatively quick regression of fibrosis, this usually occurred only at a time when there was already evidence of engraftment as judged from a rise in peripheral granulocyte counts.

We conclude that the presence of marrow fibrosis per se should not preclude any patient with a suitable donor from consideration for marrow transplantation. Fibrosis often is mild and in most instances does not impair prompt engraftment. However, patients with severe fibrosis may be at an increased risk of failure of sustained engraftment.

REFERENCES

1. Thomas ED, Storb R, Clift RA, Fefer A, Johnson FL, Neiman PE, Lerner KL, Glucksberg H, Buckner CD: Bone marrow transplantation. N Engl J Med 292:832;895, 1975

2. O'Reilly RJ: Allogeneic bone marrow transplantation: Current status and future directions. Blood 62:941, 1983

3. Storb R, Prentice RL, Thomas ED, Appelbaum F, Deeg JH, Doney K, Fefer A, Goodell BW, Michaelson E, Stewart P, Sullivan KM, Witherspoon RP: Factors associated with graft rejection after HLA-identical marrow transplantation for aplastic anemia. Br J Haematol 55:573, 1983

4. Thomas ED: Marrow transplantation for malignant diseases. J Clin Oncol 1:517, 1983

5. Doney K, Buckner CD, Sale GE, Ramberg R, Boyd C, Thomas ED: Treatment of chronic granulocytic leukemia by chemotherapy, total body irradiation and allogeneic bone marrow transplantation. Exp Hematol 6:738, 1978

6. McGlave PB, Brunning RD, Hurd DD, Kim TH: Reversal of severe bone marrow fibrosis and osteosclerosis following allogeneic bone marrow transplantation for chronic granulocytic leukemia. Br J Haematol 52:189, 1982

7. Oblon DJ, Elfenbein GJ, Braylan RC, Jones J, Weiner RS: The reversal of myelofibrosis associated with chronic myelogenous leukemia after allogeneic bone marrow transplantation. Exp Hematol 11:681, 1983

8. Mehta AB, Baughan ASJ, Catovsky D, Goldman JM, Johnson SA, Galton DAG: Reversal of marrow fibrosis in acute megakaryoblastic leukemia after remission induction and consolidation chemotherapy followed by bone marrow transplantation. Br J Haematol 53:445, 1983

9. Rappeport J, Parkman R, Belli J, Levey R, Rosen F, Nathan D: Reversibility of myelofibrosis (MF) after bone marrow transplantation. Blood 52:271a, 1978

10. Rozman C, Granena A, Hernandez-Prieto M, Vela E, Brugues R: Bone marrow transplantation for acute myelofibrosis. Lancet 1:618, 1982

11. Smith JW, Shulman HM, Thomas ED, Fefer A, Buckner CD: Bone marrow transplantation for acute myelosclerosis. Cancer 48:2198, 1981

12. Wolf JL, Spruce WE, Bearman RM, Forman SJ, Scott EP, Fahey JL, Farbstein MJ, Rappaport H, Blume KG: Reversal of acute (malignant) myelosclerosis by allogeneic bone marrow transplantation. Blood 59:191, 1982

13. Bauermeister DE: Quantitation of bone marrow reticulin—a normal range. Am J Clin Pathol 56:24, 1971

14. Appelbaum FR, Storb R, Ramberg RE, Shulman HM, Buckner CD, Clift RA, Deeg HJ, Fefer A, Sanders J, Stewart P, Sullivan K, Witherspoon R, Thomas ED: Allogeneic marrow transplantation in the treatment of preleukemia. Ann Int Med 100:689, 1984

15. Deeg HJ: Marrow transplantation in preleukemia. JNCI (in press)

16. Thomas ED, Storb R: Technique for human marrow grafting. Blood 36:507, 1970

17. Storb R, Epstein RB, Graham TC, Thomas ED: Methotrexate regimens for control of graft-versus-host disease in dogs with allogeneic marrow graft. Transplantation 9:240, 1970

18. Deeg HJ, Storb R, Thomas ED, Flournoy N, Kennedy MS, Banaji M, Appelbaum FR, Bensinger WI, Buckner CD, Clift RA, Doney K, Fefer A, McGuffin R, Sanders JE, Singer J, Stewart P, Sullivan KM, Witherspoon RP: Cyclosporine as prophylaxis for graft-versus-host disease: A randomized study in patients undergoing marrow transplantation for acute nonlymphoblastic leukemia. Blood 66:1325, 1985

19. Storb R, Deeg JH, Thomas ED, Appelbaum FR, Buckner CD, Cheever M, Clift RA, Doney K, Flournoy N, Kennedy M, McGuffin RW, Sale GE, Sanders J, Singer J, Stewart P, Sullivan KM, Witherspoon RW: A controlled trial of cyclosporine versus methotrexate for prophylaxis of graft-versus-host disease after marrow transplantation for chronic myelocytic leukemia. Blood 66:698, 1985

20. Deeg HJ, Storb R, Appelbaum FR, Kennedy MS, Graham TC, Thomas ED: Combined immunosuppression with cyclosporine and methotrexate in dogs given bone marrow grafts from DLA-haploidentical littermates. Transplantation 37:62, 1984

21. Martin PJ, Hansen JA, Buckner CD, Sanders JE, Deeg HJ, Stewart P, Appelbaum FR, Clift R, Fefer A, Witherspoon RP, Kennedy MS, Sullivan KM, Flournoy N, Storb R, Thomas ED: Effects of in vitro depletion of T-cells in HLA-identical allogeneic marrow grafts. Blood 66:664, 1985

22. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE: Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. Transplantation 18:295, 1974

23. Kennedy, MS, Deeg HJ, Storb R, Doney K, Sullivan KM, Witherspoon RP, Appelbaum FR, Stewart P, Sanders J, Buckner CD, Martin P, Weiden P, Thomas ED: Treatment of acute graft-versus-host disease after allogeneic marrow transplantation: A randomized study comparing corticosteroids and cyclosporine. Am J Med 78:878, 1985

24. Peto R, Pike MC, Armitage P, Breslow NE, Cox DR, Howard SV, Mantel N, McPherson K, Peto J, Smith PG: Design and analysis of randomized clinical trials requiring prolonged observation of each patient. Br J Cancer 3:1, 1977

25. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Ass 53:457, 1958

26. Kalbfleisch JD, Prentice RL: The Statistical Analysis of Failure Time Data. New York, John Wiley, 1980

27. Matthews DE, Farewell V: Using and Understanding Medical Statistics. New York, S. Karger, 1985

28. Beatty PG, Clift RA, Mickelson EM, Nisperos BB, Flournoy N, Martin PJ, Sanders JE, Storb R, Thomas ED, Hansen JA: Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 313:765, 1985

29. Tavassoli M, Friedenstein A: Hematopoietic stromal microenvironment. Am J Hematol 15:195, 1983

30. De la Chapell A, Vuopio P, Borgstrom GH: The origin of bone marrow fibroblasts. Blood 4:783, 1973

31. Fialkow PJ, Jacobson RJ, Papayannopoulos T: Chronic myelocytic leukemia. Clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med 63:125, 1977

32. Weisenburger DD: Acute myelofibrosis terminating as acute myeloblastic leukemia. Am J Clin Pathol 78:128, 1980

33. Castro-Malaspina H, Rabellino EM, Yen A, Nachman RL, Moore MAS: Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. Blood 57:781, 1981

34. Nowell P, Jensen J, Gardner F, Murphy S, Chaganti RSK, German J: Chromosome studies in "preleukemia." III. Myelofibrosis. Cancer 18:1873, 1976

35. Ross R, Vogel A: The platelet derived growth factor. Cell 14:203, 1980

Absence of Oncogene Amplifications and Occasional Activation of N-*ras* in Lymphoblastic Leukemia of Childhood

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To examine whether determination of (1) the copynumber or restriction pattern of certain oncogenes or (2) the mutational activation of the N-*ras* gene might contribute to the risk classification of acute lymphoblastic leukemia of childhood (ALL), we investigated DNA isolated from lymphoblasts of untreated patients. Restriction enzyme analysis of cellular oncogenes was performed on DNA of 25 patients. No rearrangements could be demonstrated within or near the genes c-*myc*, c-*myb*, c-*abl*, *bcr*, c-Ki-*ras*, and N-*ras*. No amplifications of these genes nor of N-*myc* or c-Ha-*ras* were present. Eight of 21 patients were heterozygote for "rare" Ha-*ras* allelic restriction fragments that have been associated with an increased risk of developing a malignancy. These patients were clinically indistinguishable from patients lacking these fragments. The breakpoint cluster region (*bcr*) that is rearranged in all patients with Philadelphia chromosome positive chronic myeloid leukemia, was normal in all cases, including at least one patient with Philadelphia chromosome positive ALL. A 2.8 kb HindIII fragment of a hitherto unknown gene or pseudogene related to v-*myb* probably derives from the Y chromosome. Nineteen patients were examined for point mutations in the N-*ras* gene, using a novel synthetic oligonucleotide hybridization assay. In two patients activating point mutations were present, both in positions 1 of the 12th codon. Both patients were somewhat older than the others (16 and 11 years), had L2 morphology, and were shown to have high growth fractions of tumor in their bone marrow.

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THE rapidly expanding knowledge about oncogene activation in experimental tumor systems (for review see reference 1) has not been paralleled by a similar increase in data on oncogenes in uncultured human malignancies. Though essential for future clinical applications, the study of adequately sized series of patients is hampered by several factors, including the limited availability of fresh human tumor specimens, the time-consuming and laborious nature of techniques such as the NIH/3T3 focus assay, and the inadequacy of current methodology to reliably detect activated non-*ras* oncogenes.

ALL of childhood may present with widely varying clinical and biologic features, such as L1, L2, or L3 morphology, absence or presence of gross lymphadenopathy and organomegaly, high or low S-phase percentages, or B-, T-, or null-cell phenotype. A significant proportion of patients can be shown to have hyperdiploid disease in which the median cellular DNA content is increased, often by a factor of 1.2.[2] The surplus of chromosomal material may consist of additional copies of whole chromosomes and of recognizable and unrecognizable chromosomal fragments. Although double-minute chromosomes or homogeneousy staining regions are known to be very rare in the karyotype of children with ALL, it is conceivable that amidst this genetic instability low-grade amplification or activating rearrangements of certain (from case to case varying) genes may occur that provide a selective growth advantage and contribute to the particular clinical or biologic characteristics of the patient's disease. The known oncogenes may be considered likely candidates for such a role.

The incidence of activation of known oncogenes in fresh acute lymphoblastic leukemia (ALL) cells is unclear. Hybridization of cellular RNA with oncogene probes under low stringency conditions[3] indicates that many (proto)oncogenes may be expressed in ALL, but no definite pattern is evident. Other studies have reported varied expression of several oncogenes in isolated cases, including *fes* and *myb*,[4] *myc* and *abl*,[5] *src*,[6] and *erbB*.[7] In a recent study[8] that utilized RNA from normal lymphocytes, both before and after PHA (phytohaemagglutinin) stimulation as controls, no expression of *scr* and *erb* was found. In a series of 32 high-risk ALL patients (WBC counts >5 × normal), considerably increased expression of c-*myc* was found in six cases;[9] in none of these, amplification or rearrangement of the *myc*-locus could be demonstrated. One patient with L3 (Burkitt type) ALL has been reported to have a translocated and rearranged *myc* gene, in association with a t(8;14) translocation.[10]

Studies using the NIH/3T3 transfection assay have implicated members of the *ras* family in ALL. Transforming N-*ras* has been found in a number of T-cell lines, and transforming Ki-*ras* was identified in a single line.[11,12] One abstract reported identification of N-*ras* as the transforming gene in primary transfectants of all of six DNAs of patients with common ALL and in a single case of undifferentiated ALL that was examined.[13] These data seem to argue in favor of an ethiologic role of N-*ras* in ALL or in one of the subgroups of the disease.

We employed restriction enzyme analysis to screen for amplification or rearrangement of cellular oncogenes in 25 samples of untreated acute lymphoblastic leukemia of childhood. In addition, we used a novel and highly specific oligonucleotide hybridization assay[14,15] to detect activating point mutations in the N-*ras* genes of 19 patients. Special care was taken to include samples of high-risk (WBC > 50,000/mm^3), standard-risk (WBC < 50,000/mm^3), and low-risk (hyperdiploid) patients in the study. To assess

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whether the findings might be of use in refining the risk classification of ALL, the alterations were correlated to clinical features at presentation, to cytogenetic and cell kinetic parameters, and to survival.

PATIENTS, MATERIALS, AND METHODS

Patients. The diagnosis of ALL was based on microscopic examination of peripheral blood and bone marrow, using standard criteria, and was confirmed by cytochemic methods (Sudan Black, peroxidase, Periodic acid-Schiff, acid phosphatase, and nonspecific esterase stains) and immunologic markers, including common ALL antigen, E-rosettes, human T-lymphocyte antigen (HTLA), terminal deoxynucleotidyl transferase (TdT), Ia-like antigens, and cytoplasmatic and membrane immunoglobulin determinations. On the basis of these data, each patient was assigned to one of the following diagnostic categories: (1) common ALL, (2) undifferentiated ALL, (3) T-cell ALL, (4) B-cell ALL, and (5) pre-B–cell ALL. Patients with T-cell leukemia and a mediastinal mass were classified as having non-Hodgkin's lymphoma and were not included in the study.

All patients were below 17 years of age at the time of diagnosis. None had received prior chemotherapy or radiotherapy, with one exception, who had been treated with a single administration of vincristine, prednisone, and doxorubicin. Some patient characteristics are listed in Table 1.

Samples. Mononuclear cells of blood and/or bone marrow of patients with ALL were obtained by standard Ficoll-Hypaque density centrifugation, resuspended in alcohol 70%, and stored for a period of two days to 6 years.

Eighteen of the samples were pretreatment samples from the time of diagnosis and contained 20 million or more cells, of which at least 66% were lymphoblasts. These were selected for DNA extraction. To increase the size of the study, seven additional samples were analyzed that satisfied the same criteria, but that had been stored under liquid nitrogen (Table 1). The median blast count of the samples was 94% (range 66% to 100%).

Flow cytometry. DNA per cell histograms of both peripheral blood and bone marrow saamples were recorded as described previously.[16]

Cytogenetic studies. The majority of patients were examined by direct chromosome analysis of bone marrow samples.[17] Other samples of marrow or peripheral blood were first cultured for 24 to 48 hours and colcemid was added before harvesting, as described.[2]

DNA isolation and molecular hybridization. High molecular weight DNA was isolated as described elsewhere.[18] The DNA solutions were digested with restriction endonucleases (EcoR1, HindIII, BamHI, BglII, PstI, MspI, and HpaII. all obtained from Boehringer Mannheim, Mannheim, FRG) with an enzyme:substrate ratio of 4:1, using incubation buffers as specified by the manufacturer. Test gels of the samples with codigested bacteriophage lambda were run to ascertain the completeness of the digestion and to visually compare the DNA concentrations in each sample. The concentrations were additionally assayed by fluorometry using the dye Hoechst 33258.[19]

Ten micrograms of each DNA were separated on a 0.6% agarose gel and blotted to nitrocellulose paper (Schleicher and Schuell) as described.[20] Care was taken to layer an equal amount of DNA into each slot of the gel. DNAs of healthy controls were treated in the same way and blotted to each filter for comparison of restriction patterns.

The molecular probes were obtained as gifts from the following investigators: v-*myc* from J M Bishop,[47] v-*myb* "VM2" from G Gonda,[22] v-K-*ras* "HiHi3" from R W Ellis,[23] N-*ras* "probe B" from A Hall,[24] c-H-*ras* (PEJ) from M Wigler,[25] v-*abl* "AB3SUB3" from S P Goff,[26] and the *bcr* 3" and 5" probes from G Grosveld.[27,28]

Table 1. Characteristics and Evaluability of 25 ALL Patients

Patients	
male	9
female	16
diploid*	17
hyperdiploid	7
FAB† class	
L1	14
L2	7
L3	1
Subtype	
common ALL	9
undifferentiated	7
T-cell	5
B-cell	0
pre B cell	2
standard risk‡	6
high risk	16
Evaluable for:	
flow cytometry	25
survival	22
immun. markers	22
presenting clinical features	22

*Cellular DNA content, as determined by flow cytometry.

†FAB, French-American-British classification of acute leukemias.

‡High-risk patients were considered: patients with WBC counts > 50,000/mm^3 or T-cell ALL. The median survival of "high risk" patients was 26 months, while that of "standard risk" patients was over 65 months.

The filters were hybridized to ^{32}P-labeled nick translated probes, as described.[18] The filters were subsequently washed five times in 3 × SSC (1 × SSC is 0.15 mol/L NaCl; 0.015 mol/L trisodium citrate), 0.1% SDS (sodium dodecyl sulphate) at 65 °C. The final washing conditions were of low-to-moderate stringency for the viral oncogene probes (3 × SSC, 0.1% SDS), and moderate stringency for the cellular probes (1 × SSC, 0.1% SDS), both at 65 °C. The hybridized filters were subjected to autoradiography at −70 °C using Kodak XS-1 film and a DuPont Cronex intensifying screen (DuPont, s'Hertogenbosch, the Netherlands).

To avoid misinterpretation of intensity variations as a result of partial DNA degradation, each filter was hybridized to at least one additional probe, allowing comparison of relative signal intensities. The restriction patterns were interpreted using published maps of c-*myc*,[9,30] c-*myb*,[31,32] c-*abl* and *bcr*,[27,28] c-Ki-*ras*,[33,34] and N-*ras*.[35,24]

N-ras *oligonucleotide hybridization assay.* Screening for and characterization of point mutations in or near codons 12 and 61 of N-*ras* genes was carried out as described elsewhere.[14,15] Briefly, 15 micrograms of PstI digested DNA isolated from lymphoblasts were separated on a 0.5% agarose gel. The denatured and subsequently dried gels were hydridized to synthetic 20-mer oligonucleotide probes that correspond to regions of the human N-*ras* gene, localized around codons 12 or 61. Using these probes, single basepair substitutions in these regions can be detected, by virtue of the fact that fully matched oligomer DNA hybrids are more stable than one-basepair mismatched ones. The latter can be selectively removed by washing the gel membranes at carefully controlled temperatures. DNAs of the cell lines HT 1080 (mutation at position 61-1), HL 60 (mutation at position 61-2), RD 301 (mutation at position 61-3), and MOLT 4 (mutation at position 12-1) were included as positive controls. As a positive control for a codon 13 mutation, DNA isolated from fresh myeloblasts from a patient with acute myeloid leukemia (mutation a position 13-2, unpublished) was also included.

First, all samples were hybridized to the two "wild type" oligomer probes (that is, to an oligomer probe corresponding to the unmutated N-*ras* sequence centering around either codon 12 or codon 61). All samples showing decreased signal intensity at autoradiography after one "wild type" hybridization as compared to other samples or controls, or as compared to the second "wild type" hybridization of the same sample, were further investigated. For positive identification of the point mutation, full sets of oligonucleotide probes corresponding to each possible basepair change in codons 12, 13, or 61 were employed. This strategy confirms the presence of point mutations as suggested by the "wild type" hybridizations, and additionally provides sequence information with respect to these codons.

Clinical correlations. The findings of the restriction enzyme analyses, the H-*ras* allelic restriction fragment size determinations and the N-*ras* activation assay were correlated with pertinent clinical and laboratory parameters, using a microcomputer and a program devised by one of us (S.R.). These parameters included age, sex, WBC, percentage of blasts in bone marrow and peripheral blood, modal DNA per cell content and percentage of S-phase cells in bone marrow and peripheral blood (as determined by flow cytometry), blast morphology (French-American-British classification), cytochemic stains, immunologic markers (see "Patients"), cytogenetic data, clinical features at diagnosis (presence or absence of fever, hemorrhagic manifestations, CNS involvement, degree of splenomegaly, lymphadenopathy, hemoglobin, platelet count), inducability of complete remission with first-line chemotherapy, duration of first remission, and survival.

RESULTS

Samples. A total of 25 patients, aged 5 months to 17 years (median 4 years) was studied, with a wide variety of presenting features. The median WBC count was 1,200/mm^3 (range 0.2 to 1,500,000/mm^3). Further characteristics are listed in Table 1. Eighteen samples (13 × bone marrow, 5 × peripheral blood) had been stored in 70% ethanol, seven samples (4 × bone marrow, 3 × peripheral blood) had been stored under liquid nitrogen. The yield of DNA from the 70% ethanol fixed samples was 5.4 ± 2.4 [SD] pg DNA per cell. There was no correlation with the duration of the storage period, indicating the absence of DNA degradation in the presence of 70% ethanol at 4 °C.

Myc. Restriction analysis of the c-*myc* gene with EcoR1, HindIII, BamHI, and BglI, using the viral *myc* gene as a probe yielded no evidence of either amplifications of c-*myc* or rearrangements within a 28.5 kb region containing the gene. The hybridization conditions used should also allow detection of an amplified N-*myc* gene[36] as a 2.0 kb Eco R1 fragment. No band of this size was visualized, indicating the absence of high copy numbers of N-*myc*.

MYB. Digestions with EcoR1, HindIII, BamHI, and Bg1II were hybridized to the v-*myb* probe. As with *myc*, no evidence of amplification or rearrangement within a 21.5 kb region encompassing the c-*myb* gene was apparent. In the EcoR1 digestion, however, a variable 1.7 kb band is visible that probably represents an additional EcoR1 site, cleaving the normal 2.6 kb EcoR1 fragment in two smaller fragments of 1.7 and 0.9 kb. The presence or absence of this EcoR1 site did not correlate with any of the clinical parameters.

Due to the relatively low stringency conditions used with the viral probe, several bands not accounted for by published restriction maps[31,32] were seen. The patterns of these extra bands are relatively constant between patients, but in the HindIII digestion, two clear and comparatively intense variable bands were observed, of 2.8 and 2.3 kb, respectively (Fig 1). The 2.8 kb band was seen in 9/9 male DNAs (including two controls) and in 0/14 female DNAs, strongly suggesting the presence of a *myb*-like sequence on the human Y chromosome. Due to low signal intensity, four DNAs were not evaluable for the presence of this band, including that of a male patient missing a Y chromosome in the leukemic blasts (BL, Table 2).

Further variations in intensity of non–c-*myb* bands were noted in the other digestions. Eg, 13/25 evaluable DNAs lacked a faintly hybridizing 2.0 kb Bg1II fragment that was present in both controls. Clinical correlations were absent.

RAS. N-*ras* was analyzed by hybridizing restriction enzyme digestions with EcoR1, HindIII, BamHI, and Bg1II. In 25 patients with ALL, no amplification was detectable, and there were no rearrangements in a region of 27 kb containing the N-*ras* gene.

The findings with respect to the c-Ki-*ras*2 gene and the Ki-*ras*1 pseudogene were also negative. No rearrangements were detectable in a 27.8 kb region encompassing exons 1 to 4A of the K-*ras*1 gene.[34] A restriction enzyme analysis of the two c-Ha-*ras* loci was not done, but the BamHI digestions (see below) yielded no evidence of amplification.

ABL and BCR. A 35 kb region encompassing the c-*abl* gene was analyzed using restriction enzymes EcoR1, HindIII, BamHI, and Bg1II and a viral *abl* probe. Again, no cases of *abl* amplification were noted and there were no evident rearrangements. In a few DNAs, however, clear and intense extra bands were present in the HindIII (1 ×) or Bg1II (2 ×) digestions. The patients of whom the samples were obtained did not display any unusual clinical features. Since the abnormalities were limited to single digestions, they probably represent polymorphisms rather than rearrangements of the gene.

The existence of *abl*-related sequences is suggested by the bands not accounted for by published restriction maps of the human c-*abl* locus (Fig 1).[27,28] Five DNAs showed an addi-

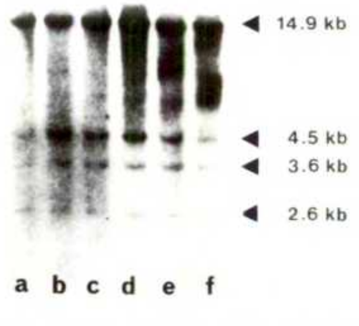

Fig 1. *abl*-related sequences. Genomic DNAs were digested with HindIII and hybridized to a viral *abl* probe. The fragments of 14.9, 4.5, and 2.6 kb originate from the c-*abl* gene. The 3.6 kb fragment is thought to correspond to a c-*abl*–like (pseudo)gene. Lanes a–e: ALL patients; lane f: normal control.

Table 2. Karyotypic Features of 16 ALL Patients

	Abnormal Cells (%)	Cytogenetic Findings
5 patients	no abnormalities	—
patient JVo	96	46,XY/52,XXY,+9,+14, +18,+21,+21
patient BL	100	45,X/45,X,del(9)(p21)/ 46,XY,del(9)(p21)
patient LJ	70	46,XX/57,XXXX,+4,+6,+8, +10,+14,+17,+18, +21,+21
patient EB	31	46,XX/55−56(+21)
patient WP	23	46,XX/46,XX,t(12;13) (p11;q11)
patient RD	93	47,XY,+marX,t(9;22)(q34; q11),del(10)(p13)
patient YS	34	46,XX/46,XX,t(1;11) (p22;q23)
patient JH	50	46,XX/45,XX,del(13) (q14),−20
patient CP	100	46,XX,t(4;11)(q21;q23)
patient SE	39	46,XX/46,XX,t(3;7;14)(q13; p13;q32)/45,XX,−17
patient LK	58	46,XX/80−91/83,XXX,+1, +1,+2,+3,+4,+5,+6, +6,+7,+8,+8,der(9) t(9;10) (p22;q11), −10,+11,+12,+12, +12,+13,+13,+14, +15,+15,+16,+16, +17,+17,+18,+19, +19,+20,+20,+21, +21,+22,+22,+22, +mar,+mar,+mar.

Detailed cytogenetic analysis of patients EB and LK was not possible. For LK the cytogenetic findings are given for a single cell but the der(9)t(9;10) could be recognized in every metaphase.

tional 1.5 kb BglII fragment but had no unexpected clinical features in common.

Rearrangements of the *bcr* locus were tested for using HindIII, BamHI, and BglII digestions, and two probes, one specific for the 5′ part of the *bcr* region, the other specific for the 3′ part.[27,28] This combination of enzymes and probes should detect rearrangements within the *bcr* region and additionally in a region of 9 kb upstream and 1 kb downstream of the *bcr*. In the 25 ALL samples investigated, not a single case of *bcr* rearrangement was noted. The one sample from a Philadelphia chromosome positive patient (cytogenetic features: 47 XY, + marker X, t(9;22)(q34;q11), del(10)(p13)) notably showed no *bcr* locus abnormalities (not shown).

*Ha-*ras *allelic restriction fragments.* The size of the BamHI fragment of the Ha-*ras*1 genes was determined in 21 patients (Table 3). The four most frequently occurring sizes were 6.9, 7.5, 8.0, and 8.3 kb, which is in agreement with the data of Krontiris et al.[29] In eight samples "rare" Ha-*ras* allelic restriction fragments were detected. The presence of such a fragment was not clearly associated with any of the clinical features. The frequency of occurrence of "rare fragments" was highly significantly increased over that in a large healthy control population published elsewhere, viz 8 out of 230 alleles [P $<<$ 0.001, chi-square test].[29] However, since the relative frequency of certain *ras* alleles may differ between geographic localizations, this statistic significance should be interpreted with caution.

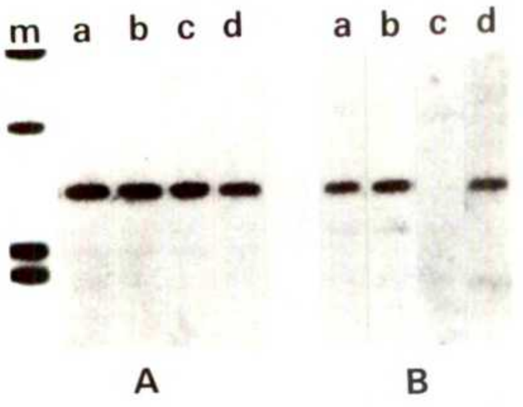

Fig 2. **N-*ras* point mutations in two ALL patients. (A): Hybridization of genomic DNAs to a synthetic 20-mer corresponding to the "wild type" N-*ras*-sequence, containing the normal codons 12 and 13. (B): Hybridization of the same DNAs to a mixture of three synthetic 20-mers containing the same codons, but each with one of the three possible point mutations in position 1 of codon 12. Lane a: patient LK; lane b: patient J Vi; lane c: ALL patient with normal N-*ras* gene (negative "control"); lane d: MOLT-4 DNA (positive control); lane m: size marker (lambda × HindIII).**

*Oligonucleotide hybridization analysis of N-*ras. Of the 19 patients examined for activating point mutations of the N-*ras* gene, two (patients LK and JVi) were positive (Fig 2). Both had point mutations in codon 12, 1st position, of one allele, the other being normal. Point mutations in the other sites that may lead to transforming activity, codons 13 and 61, were absent. The limited quantity of DNA did not allow the determination of the substituting aminoacid for position 12 in the p21 protein.

Patient L.K. was an 11-year-old girl who presented with

Table 3. Allelic Restriction Fragments (BamHI) of the Ha-*ras* Locus of 21 ALL Patients

Fragment Size	No. of Alleles	%
Common		
6.9 kb	19	45.2
7.5 kb	6	14.3
8.0 kb	9	21.4
8.3 kb	0	0
Rare		
7.1 kb	3	7.1
7.4 kb	2	4.8
7.6 kb	1	2.4
7.9 kb	1	2.4
8.5 kb	1	2.4

DNA, isolated from leukemic lymphoblasts and digested with BamHI, was electrophoretically separated on a 0.6% agarose gel, and blotted to nitrocellulose. DNA isolated from the same samples was digested with MspI and HpaII, separated on a 1.5% agarose gel as described[28] and blotted to nitrocellulose. The filters were hybridized to a radioactively labeled H-*ras* probe.

fever and moderate splenomegaly, anemia (hemoglobin 5.6 g/L), and thrombocytopenia (45,000/mm^3). The WBC count was 12,700/mm^3, with 77% lymphoblasts of L2 morphology. Since immunologic studies showed CALLA negativity and B- or T-cell markers were undetectable, she was classified as having undifferentiated ALL. Flow-cytometric examination showed S-phase percentages of 8.0% for peripheral blood and 13.5% for bone marrow. The malignant cells were nearly tetraploid. This was confirmed by karyotyping, which disclosed the presence of 80 to 91 chromosomes per cell. A complete remission was induced with vincristine, prednisone, and l-asparaginase, that was maintained for a duration of 23 months. Subsequently, a CNS relapse developed in spite of previous prophylactic radiotherapy and intrathecal administration of methotrexate. The patient eventually died, 30 months after the original diagnosis.

Patient J.Vi. was a 16-year-old male who presented with fever and hemorrhage. There was gross lymphadenopathy and the spleen was palpable 8 cm's below the costal margin. The hemoglobin was 9.8 g/L, the platelet count was 40,000/mm^3, the WBC count was 120,000/mm^3. 92% of the latter were lymphoblasts with L2 morphology that proved to be CALLA positive without B- or T-cell markers. The patient was classified as having common ALL. Flow cytometry showed S-phase percentages of 4% and 12.5% for blood and bone marrow, respectively, and suggested diploid disease. Cytogenetic examination was not successful. A complete remission was induced with vincristine, prednisone, and l-asparaginase, and was maintained over 13 months. The patient died 16 months after the original diagnosis from massive intraabdominal hemorrhage, while receiving remission induction chemotherapy. Autopsy showed a large tear in the splenic capsule.

DISCUSSION

Amplification is a well-documented mechanism of activation of oncogenes, and has been reported in a number of fresh human tumors, including N-*myc* in neuroblastoma,[37,38] retinoblastoma,[36] and accelerated phase of chronic myeloid leukemia,[39] c-*myb* in acute myeloid leukemia,[31] and c-Ha-*ras* in bladder carcinoma.[40,41] Amplifications of these genes, and also of c-*abl*, c-*erbB*, N-*ras*, and Ki-*ras*, have been encountered in cell lines,[1,42] but since oncogen amplifications may arise during serial passage in vitro,[43] the relevance of these observations for malignancies in vivo is uncertain.

Even oncogene amplification in fresh tumor DNA should be interpreted with caution. Chemotherapy may lead to the appearance of double-minute chromosomes (DM) or homogeneously staining regions (HSR)[44] that represent large regions of amplified DNA. It is conceivable that cellular oncogenes may be (co-)amplified in these structures, having been unamplified in the original tumor. On the other hand, while DM chromosomes and HSRs are usually associated with high copy numbers of certain genes, less extreme amplifications may well occur without these structures.[45] We therefore screened for the presence of low-grade amplifications in untreated patients.

In 25 patients with ALL of childhood, no amplification of c-*myc*, N-*myc*, c-*myb*, c-*abl*, N-*ras*, Ki-*ras*, or Ha-*ras* appeared to be present. Furthermore, no rearrangements within or near the c-*myc*, c-*myb*, c-*abl*, Ki-*ras*, or N-*ras* genes could be shown. It is of interest that amplifications and rearrangements are also absent in the hyperdiploid subtype of ALL that might be considered a more likely candidate for such changes than diploid disease. Patients with hyperdiploid disease generally present with comparatively low WBC counts.[2] Since bone marrow aspiration is usually required to obtain a sufficient number of cells for nucleic acid extraction, these patients are usually not included in reported series.

The washing of the filters at low-to-moderate stringency following hybridization allowed the detection of several bands at autoradiography that represent sequences homologous to c-*abl* and c-*myb*, but that are not part of these genes. The existence of c-*abl*–related genomic sequences has recently been reported,[46] that yield a 3.0 kb fragment in HindIII digestions. Our results appear to confirm these data 3.6 kb bands showing no remarkable variability in intensity were present (Fig 1) that may well correspond to these new genes or pseudogenes.

Rare allelic restriction fragments of the Ha-*ras* locus were found in eight of 21 patients. The diversity in fragment sizes is known to be a result of a variable number of tandem repeat sequences, located 3′ to the Ha-*ras* locus.[29] These variable tandem repeat sequences (VTRs) come in four "common" sizes (Table 3) and in a number of "rare" sizes. The increased frequency of these "rare" allelic restriction fragments in cancer patient DNAs has led to the suggestion that restriction fragment length polymorphisms at this locus will be useful in the assessment of individual risk of tumor development.[29] Preliminary evidence suggests that one of the "rare" BamHI-fragments, sized 7.8 kb, may be specifically associated with myelodysplasias and acute leukemia.[47] This fragment, however, was not encountered in one of our patients.

It is conceivable that the cancer-associated alleles might in some way affect the biologic behavior of the tumor or predispose for the development of malignancies with particular growth characteristics. To analyze this, we compared the clinical data of eight ALL patients with "rare" alleles to those of 13 patients without. The two groups were strikingly similar with respect to all clinical and laboratory features, including FAB classification, immunologic markers, S-phase percentage, survival, and other parameters (see Materials and Methods). The presumed cancer-susceptibility appears to be nonspecific for certain subtypes of ALL. Determination of Ha-*ras* allelic restriction fragment length therefore seems to be of little use in individual ALL patients.

The finding that the break cluster region (*bcr*) in the single patient with Philadelphia chromosome positive ALL was not rearranged, indicates that the breakpoint on chromosome 22 was not located at the usual site for Philadelphia chromosomes in CML. Groffen et al[28] found rearrangements of the *bcr* in all of 19 Philadelphia chromosome positive CML patients and in none of two Ph-negative patients. The absence of *bcr* rearrangement is therefore at least unusual, and it is tempting to speculate that this may reflect a basic difference between Philadelphia chromosomes in CML and ALL. The view is strengthened by the absence of *bcr* rearrangement in a second patient with Ph-positive ALL recently reported by us.[48] More patients will have to be investigated to provide an answer to this clinically and fundamentally important question.

This report shows that the oligonucleotide hybridization assay for *ras* activation represents an adequate tool to conclusively demonstrate activation of *ras* oncogenes in sizable patient series. The assay requires comparatively small amounts of DNA and can therefore be used with small samples of cells. Furthermore, the assay is less laborious and considerably less time-consuming than the NIH/3T3 assay and can provide the exact sequence of the mutated codon.

Since of most patients only small amounts of DNA were available, we limited the application of the assay to the N-*ras* gene. Of the *ras* family, this gene seems to be particularly associated with leukemias.[15] It has repeatedly been shown to be activated in ALL,[11-13] whereas K-*ras* has only been detected in a single ALL cell line and Ha-*ras* has never been associated with ALL.

Mutational activation of N-*ras* was found in only two of 19 patients. Similar figures of approximately 10% are surprisingly uniform for several human tumors that are associated with *ras* activations, including Non-Small Cell Lung Cancer,[49] breast cancer, and colon cancer.[50] It therefore seems reasonable to assume that a *ras* oncogene may render a selective growth advantage to a subpopulation in which it is activated, rather than being essential for malignant transformation or for maintenance of the malignant phenotype. If this assumption were true, one might expect to find subtle or less subtle clinical or biological differences between N-*ras* positive and N-*ras* negative malignancies.

The number of N-*ras* positive patients in our study is clearly too small to allow statistical analysis of its clinical or biological impact in ALL. The unfavorable courses of both patients are certainly not surprising, since high-risk patients are overrepresented in this study. Some common features may however be of interest. In both patients, the activating mutation was in codon 12, whereas in acute myeloid leukemia, codon 13 seems to be predominantly involved in N-*ras* activation.[15] The clinical features of the patients were quite different, but both had lymphoblasts with L2 morphology and were relatively old (16 and 11 years) as compared to the N-*ras* negative patients ($P < 0.10$, Wilcoxon test). In addition, both patients had high S-phase percentages in their bone marrow (13.5% and 12.5%), ranking second and fourth of the 17 patients in whom both N-*ras* activation and S-phase percentage were determined. Further study is required to assess whether or not N-*ras* activation in ALL is associated with L2 morphology, high growth fractions, or both, and whether N-*ras* activation is more common in adults than in children with ALL.

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REFERENCES

1. Varmus HE: The molecular genetics of cellular oncogenes. Ann Rev Genet 18:553, 1984
2. Smets LA, Slater RM, Behrendt H, Van't Veer MB, Homan-Blok J: Phenotypic and karyotypic properties of hyperdiploid acute lymphoblastic leukaemia of childhood. Brit J Haematol 61:113, 1985
3. McClain KL: Expression of oncogenes in human leukemias. Cancer Res 44:5382, 1984
4. Slamon DJ, deKernion JB, Verma IM, Cline MJ: Expression of cellular oncogenes in human malignancies. Science 224:256, 1984
5. Westin EH, Wong-Staal F, Gallo RC: Human hematopoietic cell expression of retroviral related cellular onc genes, in Magrath IT, O'Connor GT, Ramot B (eds): Pathogenesis of Leukemias and Lymphomas: Environmental Influences (Vol 27). New York, Raven Press, 1984, p 359
6. Rosson D, Tereba A: Transcription of hematopoietic-associated oncogenes in childhood leukemia. Cancer Res 43:3912, 1983
7. Roy-Burman P, Devi BG, Parker JW: Differential expression of c-*erbB*, c-*myc* and c-*myb* oncogene loci in human lymphomas and leukemias. Int J Cancer 32:185, 1983
8. Blick M, Westin E, Gutterman J, Wong-Staal F, Gallo R, McCredie K, Keating M, Murphy E: Oncogene expression in human leukemia. Blood 64:1234, 1984
9. Rothberg PG, Erisman MD, Diehl RE, Rovigatti UG, Astrin SM: Structure and expression of the oncogene c-*myc* in fresh tumor material from patients with hematopoietic malignancies. Molec Cell Biol 4:1096, 1984
10. Peschle C, Mavilio F, Sposi NM, Giampaolo A, Care A, Bottero L, Bruno M, Mastroberardino G, Gastaldi R, Testa MG, Alimena G, Amadori S, Mandelli F: Translocation and rearrangement of c-*myc* into immunoglobulin alpha heavy chain locus in primary cells from acute lymphocytic leukemia. Proc Natl Acad Sci USA 81:5514, 1984
11. Eva A, Tronick SR, Gol RA, Pierce JH, Aaronson SA: Transforming genes of human hematopoietic tumors: Frequent detection of *ras*-related oncogenes whose activation appears to be independent of tumor phenotype. Proc Natl Acad Sci USA 80:4926, 1983
12. Souyri M, Fleissner E: Identification by transfection of transforming sequences in DNA of human T-cell leukemias. Proc Natl Acad Sci USA 80:6676, 1983
13. Kitchingman GR: Transforming genes of common acute lymphocytic leukemias. Proc AACR 25:281, 1984 (abstr)
14. Bos JL, Verlaan-de Vries M, Jansen AM, Veeneman GH, van Boom JH, van der Eb AJ: Three different mutations in codon 61 of the human N-*ras* gene detected by synthetic oligonucleotide hybridization. Nucl Acids Res 12:9155, 1984
15. Bos JL, Tokzos D, Marshall CJ, Verlaan-de Vries M, Veeneman JH, van der Eb JH, van Boom JH, Janssen JWG, Steenvoorden ACM: Aminoacid substitutions at position 13 of the N-ras gene in human acute myeloid leukemia. Nature 315:276, 1985
16. Smets LA, Taminiau J, Hahlen K, de Waal F, Behrendt H: Cell kinetic responses in childhood acute nonlymphocytic leukemia during high-dose therapy with cytosine arabinoside. Blood 61:79, 1983
17. Slater RM, Philip P, Badsberg E, Behrendt H, Hansen NE, van Heerde P: A 14q+ chromosome in a B-cell acute lymphocytic leukemia and in a leukemic non-endemic Burkitt lymphoma. Int J Cancer 23:639, 1979
18. Janssen JWG, Steenvoorden ACM, Collard JG, Nusse R: Oncogene activation in human myeloid leukemia. Cancer Res 45:3262, 1985
19. Brunk CF, Jones KC, James TW: Assay for nanogram

quanitites of DNA in cellular homogenates. Anal Biochem 92:497, 1979

20. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503, 1975

21. Vennstromm B, Moscovici C, Goodman HM, Bishop JM: Molecular cloning of the avian myelocytomatosis virus genome and recovery of infectious virus by transfection of chicken cells. J Virol 39:625, 1981

22. Klempnauer KH, Gonda TJ, Bishop JM: Nucleotide sequence of the retroviral leukemia gene v-*myb* and its cellular progenitor c-*myb*: The architecture of a transduced oncogene. Cell 31:453, 1982

23. Ellis RW, DeFeo D, Maryak JM, Young HA, Shih TY, Chang EH, Lowly DR, Scolnick EM: Dual evolutionary origin for the rat genetic sequences of Harvy murine sarcoma virus. J Virol 36:408, 1980

24. Hall A, Marshall CJ, Spurr NK, Weiss RA: Identification of transforming gene in two human sarcoma cell lines as a new member of the *ras* gen family located on chromosome 1. Nature 303:396, 1983

25. Goldfarb MP, Shimizu K, Perucho M, Wigler MH: Isolation and preliminary characterisation of a human transforming gene for T24 carcinoma cells. Nature 296:404, 1982

26. Goff SP, Gilboa E, Witte ON, Baltimore D: Structure of the Abelson murine leukemia virus genome and the homologous cellular gene: Studies with cloned viral DNA. Cell 22:777, 1980

27. Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartram CR, Grosveld G: Localization of the c-*abl* oncogene adjacent to a translocation breakpoint in chronic myelocytic leukemia. Nature 306:239, 1983

28. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR, Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr*, on chromosome 22. Cell 36:93, 1984

29. Krontriris TG, DiMartino NA, Colb M, Parkinson DR: Unique allelic restriction fragments of the human Ha-*ras* locus in leukocyte and tumor DNAs of cancer patients. Nature 313:369, 1985

30. Dalla-Favera R, Martinotti S, Gallo RC, Erikson J, Croce CM: Translocation and rearrangements of the myc oncogene locus in human undifferentiated B-cell lymphomas. Science 219:963, 1983

31. Pellici PG, Lanfrancone L, Brathwaite MD, Wolman SR, Dalla-Favera R: Amplification of the c-myb oncogene in a case of human acute myelogenous leukemia. Science 224:1117, 1984

32. Leprince D, Saule S, de Taisne C, Gegonne A, Begue A, Righi M, Stehelin D: The human DNA locus related to the oncogene *myb* of avian myeloblastosis virus (AMV): Molecular cloning and structural characterization. EMBO J 2:1073, 1983

33. Nakano H, Yamamoto F, Neville C, Evans DD, Mizuno T, Perucho M: Isolation of transforming sequences of two human lung carcinomas: Structural and functional analyses of the activated c-K-*ras* oncogenes. Proc Natl Acad Sci USA 81:71, 1984

34. McGrath JP, Capon DJ, Smith DH, Chen EY, Seeburg PH, Goeddel DV, Levinson AD: Structure and organization of the human Ki-*ras* proto-oncogene and a related processed pseudogene. Nature 304:501, 1983

35. Taparowski E, Shimizu K, Goldfarb M, Wigler M: Structure and activation of the human N-*ras* gene. Cell 34:581, 1983

36. Lee WH, Murphree AL, Benedict WF: Expression and amplification of the N-*myc* gene in primary retinoblastoma. Nature 309:458, 1984

37. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM: Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science 224:1121, 1984

38. Schwab M, Ellison J, Busch M, Rosenau W, Varmus HE, Bishop JM: Enhanced expression of the human gene N-*ras* consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc Natl Acad Sci USA 81:4940, 1984

39. McCarthy DM, Rassool FV, Goldman JM, Graham SV, Birnie GD: Genomic alterations involving the c-*myc* proto-oncogene locus during the evolution of a case of chronic granulocytic leukemia. Lancet ii:1362, 1984

40. Dolnick BJ, Reznikoff CA: Amplification of the H-*ras* oncogene correlates with the malignant properties of several rodent cell lines in vitro and is associated with a clinical metastatic bladder carcinoma. Proc AACR 25:257, 1984

41. Hayashi K, Kakizoe T, Sugimura T: In vivo amplification and rearrangement of the c-Ha-*ras*-1 sequence in a human bladder carcinoma. Gann 74:798, 1983

42. Collins SJ, Groudine MT: Rearrangement and amplification of c-*abl* sequences in the human chronic myelogenous leukemia cell line K-562. Proc Natl Acad Sci USA 80:4813, 1983

43. Srivastava A, Shmookler Reis RJ, Goldstein S: C-Ha-*ras*-1 proto-oncogene amplification and expression during cellular aging in vitro. Federation Proceedings 43:2738, 1984 (abstr)

44. Schimke RT: Gene amplification, drug resistance, and cancer. Cancer Res 44:1735, 1984

45. Alitalo K, Winqvist R, Lin CC, de la Chapelle A, Schwab M, Bishop JM: Aberrant expression of an amplified c-*myb* oncogene in two cell lines from a colon carcinoma. Proc Natl Acad Sci USA 81:4534, 1984

46. White H, Young B, Goldman JM: A new human DNA-sequence related to the cellular oncogene c-*abl*. J Cell Biochem Suppl 9A:93, 1985 (abstr)

47. Krontriris TG, DiMartino NA, Colb M, Parkinson DR: Unique allelic restriction fragments of the human Ha 2ras locus in tumor and leukocyte DNA of cancer patients. J Cell Biochem 9C:1173, 1985 (suppl)

48. Rodenhuis S, Smets LA, Slater RM, Behrendt H, Veerman A: Distinguishing the Philadelphia chromosome of acute leukemia from its counterpart in chronic myelogenous leukemia. New Engl J Med 313:51, 1985 (letter)

49. Minna JD: Recent advances of potential clinical importance in the biology of lung cancer. Proc AACR 25:393, 1984

50. Schlom J: Monoclonal antibodies to tumor antigens and oncogen products in the management of human carcinomas. Proc AACR 26:381, 1985

Etiologic Aspects of Cold Agglutinin Disease: Evidence for Cytogenetically Defined Clones of Lymphoid Cells and the Demonstration That an Anti-Pr Cold Autoantibody is Derived From a Chromosomally Aberrant B Cell Clone

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This study investigated the clonal nature of cold agglutinin disease in a series of nine patients, which included the benign or idiopathic form as well as cases with an underlying lymphoma. Surface marker phenotyping and karyotypic analysis were performed on peripheral blood lymphocytes. An increased proportion of B cells was found in four cases and in three of these patients a monoclonal B cell population was identified with a mu, kappa phenotype. In the same three cases, as well as an additional patient, an aberrant karyotype was demonstrated. The cytogenetic abnormality present in all four cases included trisomy 3; two patients also had a trisomy 12. One of these four patients had a well-differentiated lymphoma and underwent a splenectomy. Splenic lymphocytes were transformed with Epstein-Barr virus and cultured en masse. Eight clones were established producing the same cold agglutinin with identical specificity as that present in the patient's plasma. Five of these clones were studied cytogenetically, and all had the same abnormal karyotype (51,XX,+3,+9,+12,+13,+18) found in peripheral blood and splenic lymphocytes. Thus, in this case, the cold reactive autoantibody was produced by the chromosomally abnormal, neoplastic clone of lymphocytes. Our findings support the view that cold agglutinin disease represents a spectrum of clonal disorders.

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CHRONIC cold agglutinin disease may be idiopathic or associated with an underlying lymphoproliferative disorder. In both instances, hemolysis is usually caused by an IgM, kappa autoantibody that binds to red blood cells at low temperature. The cold agglutinin is considered to be a monoclonal antibody because it may form a homogeneous peak on the serum protein electrophoretic pattern[1] and contains a single light chain.[2] It has been generally assumed that the cold autoantibody is derived from a clone of B cells, which may or may not have expanded to the magnitude of clinical lymphoma, but there is little real evidence in support of this conclusion. In the idiopathic form of this disorder, a clone of lymphoid cells has not been specifically demonstrated, nor has the cold autoantibody been demonstrated to be derived from a defined clone of B cells in either form of cold agglutinin disease.

We studied a series of nine patients with cold agglutinin disease, which included cases of the idiopathic form as well as patients with an underlying lymphoma. Chromosomal data and monotypic cell surface immunoglobulin of lymphocytes in four of nine patients provided definitive evidence for the clonal nature of their disorder. In one of these patients we were also able to demonstrate the origin of the cold autoantibody from a chromosomally abnormal clone of B lymphocytes.

MATERIALS AND METHODS

The study included nine patients with cold agglutinin disease in the age group of 37 to 78 years. Clinical and laboratory data of these individuals were obtained at the time of entry into this study and are summarized in Table 1. These patients had a prior history of cold agglutinin disease that ranged from 6 weeks to 10 years. All patients were considered to have idiopathic cold agglutinin disease except patients 1 and 3, in whom an underlying lymphoma was diagnosed. Three patients had evidence of organomegaly. The peripheral smear in all nine patients showed polychromasia of varying degree; spherocytosis was only present in patient 2. The hemoglobin ranged from 5.6 to 10 g/dL and was associated with an elevated reticulocyte count. The leukocyte count ranged from 5,400 to 18,600/mm^3 and was not associated with an absolute lymphocytosis (>4,000/mm^3) in any of the cases. In patients 1,2,3,4, and 8 an "M" component was noted on the serum protein electrophoretic pattern and identified as IgM, k by immunofixation.

Description of four patients with evidence for a circulating clone. Patient 1 is a 37-year-old woman with a 2-year history of cold agglutinin disease who was admitted to the hospital with a severe hemolytic episode. On physical exam, the spleen tip was palpable 3 finger breadths below the left costal margin. There was no peripheral lymphadenopathy. Splenomegaly was confirmed by ultrasound and computerized tomography scans that did not reveal abnormalities in the liver or lymphadenopathy. A bone marrow aspirate revealed erythroid hyperplasia but no increase in lymphocytes or plasma cells. Laboratory studies performed at that time are summarized in Table 1. She was treated with red blood cell (RBC) transfusions, corticosteroids, and plasma exchange. Despite 8 weeks of therapy her hemoglobin level remained below 7 g/dL, and a splenectomy was performed. Light microscopic examination of splenic tissue and two splenic hilar lymph nodes was consistent with a malignant lymphoproliferative disorder. Frozen section analysis of the spleen and one node for cell surface antigens suggested a monoclonal population of B cells which stained positively with antibodies to B1, IgM, kappa, and HLA-Dr. The patient was diagnosed as having a well-differentiated lymphoma. Clinically she did not respond to splenectomy and is presently receiving combination chemotherapy consisting of cyclosphosphamide, vincristine, and corticosteroids.

Patient 2 is a 62-year-old man who at the time of the present study, had a 2-year history of chronic cold agglutinin disease. Physical exam revealed hepatomegaly which was confirmed by liver spleen scan with a homogenous uptake of radionucleotide. A liver biopsy and bone marrow studies failed to show evidence of a lymphoproliferative disease process. Laboratory data obtained 2 years after initial diagnosis are illustrated in Table 1. The patient

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Table 1. Clinical and Laboratory Data of Nine Patients with Cold Agglutinin Disease

Patient No.	Age	Duration of Disease* (yr)	Organomegaly	Histologic Evidence for Lymphoma	Hgb (g/dL)	Ret. Ct. (%)	Titer†	Paraprotein‡†
1	37	2	splenomegaly	yes	7.4	16	512	IgM, k
2	62	2	hepatomegaly	no	7.0	12	16384	IgM, k
3	63	6	hepatosplenomegaly	yes	8.5	14	2048	IgM, K
4	78	6	—	no	7.0	18.2	8192	IgM, k
5	74	8	—	no	11	1.9	4096	—
6	70	10	—	no	5.6	10.6	4096	—
7	61	5	—	no	12	5	2048	—
8	70	5	—	no	8.4	17.6	16384	IgM, k
9	69	½	—	no	10.1	9.5	1024	—

*At the time of entry into the study.

†The values are expressed as reciprocal of titer at 4 °C against adult red blood cells.

‡The nature of the paraprotein was determined in the serum by protein electrophoresis followed by immunofixation.

continues to have chronic hemolytic anemia, which is treated intermittently with corticosteroids.

Patient 3 was admitted to the hospital with the diagnosis of hemolytic anemia and was treated for 2 weeks with corticosteroids and blood transfusions. Physical exam showed moderate hepatosplenomegaly but no peripheral lymphadenopathy. Pertinent laboratory data are shown in Table 1. A bone marrow aspirate and biopsy performed 6 weeks after diagnosis of cold agglutinin disease showed sheets of small lymphocytes consistent with lymphoma. The patient continued to have severe hemolysis and required frequent RBC transfusions. He is presently undergoing combination chemotherapy in an attempt to control the hemolytic process that is ascribed to an underlying lymphoma.

Patient 4 is a 78-year-old woman with a 6-year history of idiopathic cold agglutinin disease. During the past 3 years she has required intermittent RBC transfusions for symptomatic anemia. By physical exam, chest x-ray, and bone marrow studies the patient has no evidence of an underlying lymphoma. She was admitted to our hospital for treatment of an exacerbation of her cold agglutinin disease. Physical exam revealed no organomegaly and the extremities showed no evidence of acrocyanosis. Laboratory data are shown in Table 1. She was transfused with 2 units of packed red blood cells and was discharged with a stable hemoglobin of 9.2 g/dL. During the next 4 months she did not require additional therapy and maintains a hemoglobin of 8 to 9 g/dL.

Serology. Cold agglutinin titers were performed at 4 °C with measured quantities of group O normal adult red blood cells (RBC), I-antigen negative group O adult RBCs, group O cord RBCs, and autologous RBCs.[3] Test erythrocytes were also treated with ficin and neuraminidase according to standard methods.[3,4] The serum of all patients was also incubated with 0.01 mol/L of the IgM-reducing agent dithiothreitol (DTT) in phosphate-buffered saline and tested for residual cold autoantibody activity.[5]

Isolation of cold autoantibodies. Equal volumes of saline-washed packed red blood cells and patient serum were incubated for two hours at 4 °C with inversion of the tubes every ten minutes. The cells were centrifuged and the supernatants removed at 4 °C. The cells were washed six times with normal saline (4 °C) and a heat eluate prepared[4] at 40 °C. The cold antibody isotype was then evaluated by Ouchterlony analysis (Cooper Biomedical, Malvern, Pa).

Surface marker phenotyping of lymphocytes from peripheral blood and spleen. Lymphocytes, separated from peripheral blood (and spleen in one case) by density gradient centrifugation on Ficoll-Hypaque, were labeled by a double-antibody fluorescent staining technique. Cells were incubated for 30 minutes at 4 °C with an appropriate dilution of mouse monoclonal antibodies directed against B- or T-cell surface antigens. Antibodies used were OKIa, OKT11, OKT3, OKT4, OKT8 (Orthomune, Ortho Diagnostic Systems Inc., Raritan, NJ); B1, (Coulter Immunology, Hialeah, Fla); and antihuman IgG, IgM, IgD, kappa and lambda light chains (Becton Dickinson Co, Sunnyvale, Calif). Labeled cells were washed three times in cold phosphate staining buffer and stained with a second antibody, fluorescein conjugated goat antimouse IgG (Tago, Burlingame, Calif). Cells were washed, fixed, and analyzed by cytofluorimetry (Spectrum III, Ortho Diagnostics).

Karyotypic analysis. Mononuclear cells were cultured for 72 to 96 hours with a combination of a 1:1000 dilution of pokeweed mitogen (Gibco Laboratories, North Andover, Mass), and the phorbol ester TPA (Chemicals for Cancer Research, Eden Prairie, Minn, 0.01 to 0.05 mcg/mL) supplemented with crude preparations of interleukin-2 that contained B cell growth factor activity, as previously described.[6]

Air dried slide preparations were trypsin-Giemsa banded by standard techniques.[6] From 12 to 56 metaphases were counted, and at least three karyotype analyses were performed on each specimen. Additional karyotypic analyses were carried out when necessary to characterize chromosomally abnormal clones.

Establishment of Epstein-Barr Virus (EBV)-transformed cell lines. Splenic lymphocytes from patient 1 were isolated by Ficoll-Hypaque gradient centrifugation. Cells (10^7) were suspended in 4 mL RPMI 1640 medium, supplemented with 10% fetal bovine serum and 10 ug/mL/gentamycin. The EBV-containing supernatant of the B95-8 Marmoset cell line[7] was first freeze-thawed and subsequently filtered. One mL of this supernatant was added to the lymphocyte suspension. The lymphocytes were then cultured at 37 °C in 5% CO_2 in 95% humidified air in 25 cm^2 tissue culture flasks for 6 weeks. Karyotypic analysis was performed on the cells, and the supernatant was tested for cold agglutinin activity against untreated and ficin-treated group O normal adult RBCs. The cells were subcloned by limiting dilution in 96 well plates. Statistical clones were then again analyzed by karyotypic analysis and their supernatant tested for cold agglutinin activity.

RESULTS

Serology of cold autoantibodies. The cold agglutinin of patient 1 was not reactive against enzyme-treated RBCs and was therefore classified as anti-Pr.[8] The reactivity of the other eight patients' cold autoantibodies was enhanced with ficin-treated RBCs. The sera of patients 3,4,6,7,8, and 9 reacted stronger with adult RBCs than with cord RBC and thus demonstrated an anti-I specificity. The sera of patients 2 and 5 reacted equally strong with adult and cord RBCs.

Inactivation of cold agglutinin activity by the IgM-reducing agent dithiothreitol was observed in all patients' sera except patient 2. In the latter patient an RBC cold absorption of this patient's serum followed by heat elution identified both IgM and IgG in the eluate by Ouchterlony analysis. The presence of an IgG cold antibody or the possibility that the pathologic IgM cold antibody was, in part, monomeric[9] could explain the resistance to dithiothreitol. The cold agglutinin titers in the patients' sera ranged from 1:512 to 1:16384 (Table 1) against normal group O adult RBCs at 4 °C. All the cold agglutinins were reactive at 25 °C although weaker than at 4 °C. No cold agglutinin activity was observed at 37 °C except in patient 8 whose serum had a titer of 1:4 at 37 °C.

Surface phenotype. Surface marker phenotyping was performed on seven patients (Table 2); in the other two patients, insufficient lymphocytes were isolated for complete phenotyping. All patients demonstrated circulating T cells with a normal ratio of OKT4:OKT8 positive cells (with the exception of patient 9 where the proportion of OKT8 cells was slightly elevated). Four of the seven patients (cases 1,2,3,5) showed an increased proportion of B cells (as determined by the sum of kappa and lambda light chains ≥ 30%), and in all of these there was a distortion of the normal 2:1 kappa-to-lambda ratio suggesting the presence of a monoclonal B cell population bearing kappa light chains. The heavy chain isotype was identified as mu in three cases (patients 1,2,3); adequate data were not obtained on case 5. Patient 3 also demonstrated staining for gamma heavy chains, but this was very weak and probably extrinsic. The other cases (patients 4,7, and 9) of cold agglutinin disease showed no evidence of a circulating monotypic B cell population in the circulation.

Surface marker analysis of the splenic lymphocytes in patient 1 showed a predominance of B cells with the μ,k phenotype, comprising 68% of all splenic lymphocytes.

Karyotypic analysis. In four cases (patients 1, 2, 3, 4), a clone with an aberrant karyotype was demonstrated (Table 3). In all four cases the cells were trisomic for chromosome 3, with this being the only abnormality in two instances (patients 2, 3); in patient 1 (Fig 1) and patient 4, abnormal clones also included trisomy 12 (Table 3). A cytogenetically aberrant clone was not detected in the other cases (patients 5 through 9) of cold agglutinin disease including patient 5 where a monotypic B cell population had been identified in the circulation. Conversely, in patient 4 surface marker phenotyping of peripheral blood lymphocytes did not identify a monotypic B-cell population, while karyotypic analysis demonstrated the presence of a chromosomally abnormal clone.

Table 2. Surface Phenotype of Lymphocytes in Seven Patients With Cold Agglutinin Disease

Marker	Patient No. 1	2	3	4	5	7	9
T cell antigens							
T11	39*	47	52	79	38	58	66
T3	30	35	40	72	32	24	66
T4	21	26	28	49	15	22	29
T8	14	15	28	29	14	16	37
B cell antigens							
Ia(Dr)	49	44	30	32	44	16	28
Kappa	52	31	28	16	40	14	5
Lambda	1	3	5	10	5	5	2
IgM	48	36	25	30	‡	‡	2
IgG	1	2	19†	12	9	0	2
IgD	8	20	26	13	2	5	2
Phenotype of circulating line	u, k	u, k	u, k	—	—, k	—	—

*Results expressed as percentage of total lymphocytes.
†Very weak staining.
‡Not determined.

Characterization of EBV-transformed splenic lymphocytes. After 6 weeks of culture, cytogenetic studies of the EBV-transformed splenic lymphocytes of patient 1 revealed the presence of both eukaryotic cells as well as the clone with the aberrant karyotype 51, XX, +3, +9, +12, +13, +18 (Table 3). Through subcloning by limiting dilution, eight clones were established that also produced the cold autoantibody with Pr specificity similar to the cold antibody present in plasma of the patient; the unconcentrated supernatants of the aberrant clones reacted with group O normal adult RBC (range of titer 1:2 to 1:8) and were unreactive with enzyme-treated RBC. The cold autoantibody produced by the clones was identified as IgM, kappa by immunoelectrophoresis and displayed similar electrophoretic mobility as the paraprotein present in the plasma of the patient. Five of these clones were studied cytogenetically and all had the same abnormal karyotype with 51 chromosomes. All transformed clones expressed EBV nuclear-associated antigen, which was demonstrated by the anticomplement immunofluorescence technique.[10]

Table 3. Chromosomal Abnormalities in Four Patients With Cold Agglutinin Disease

Patient*	Tissue	Abnormal Cells†/Total Cells	Karyotype of Abnormal Clone
1	Blood	28/56	51,XX,+3,+9,+12,+13,+18
	Spleen (bulk EBV culture)	12/23	51,XX,+3,+9,+12,+13,+18
2	Blood	6/16	47,XY,+3
3	Blood	8/23	47,XX,+3
4	Blood	13/84	47,XX,+12 48,XX,+3,+12

*Cytogenetic studies on peripheral blood lymphocytes of patients 5 through 9 did not show evidence of a chromosomally abnormal clone.
†Abnormal cells – cells with karyotype of abnormal clone(s).

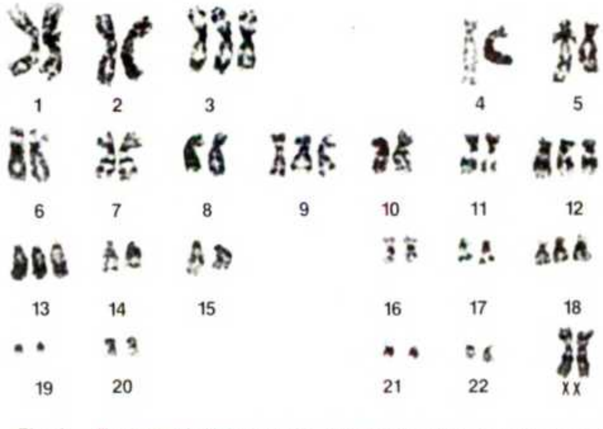

Fig 1. Representative trypsin Giemsa-banded karyotype of the abnormal clone in case 1, characterized as 51,XX,+3, +9,+12,+13,+18. This clone was identified in the peripheral blood and in EBV-stimulated cultures of spleen cells.

DISCUSSION

The majority of patients with chronic cold agglutinin disease have an associated monoclonal IgM antibody that is considered to be the pathologic cold agglutinin. The disorder is rare, having been reported primarily as isolated case reports or in small series of patients. Consequently little information is available regarding the natural history and prognosis. A recent series of a large group of patients[11] illustrated the association of monoclonal cold autoantibodies with both the idiopathic form of cold agglutinin disease as well as with a variety of lymphoid neoplasms. Based on this association it has been suggested that cold agglutinin disease represents a spectrum of disorders, ranging from a benign, autoimmune condition such as idiopathic cold agglutinin disease, to a more clearly malignant lymphoproliferative process. To date, a chromosomally aberrant clone of B cells has not been demonstrated in the idiopathic form nor in those cases of cold agglutinin disease with an underlying lymphoma. In the present study of nine patients we were able to identify a cytogenetically defined clone of B lymphocytes in 4 patients. Two of the four patients had an underlying lymphoma while the other two were considered to have the idiopathic disorder; the latter two patients had a long history of cold autoimmune hemolytic anemia and had no histologic evidence of lymphoma using conventional diagnostic methods including physical exam, computerized tomography, chest x-rays, and bone marrow studies.

It has been assumed that the cold autoantibody is produced by B lymphocytes representative of the underlying disorder, analogous to plasmacytic disorders such as multiple myeloma, where substantial evidence exists that the monoclonal immunoglobulin is produced by the malignant plasma cells.[12] Many other disease states, however, including nonlymphoid malignancies[13,14] and several autoimmune diseases[15-17] have also been described with associated M components. The specificity of the monoclonal antibodies associated with nonlymphoid neoplasms is unknown, but some authors have suggested that they may reflect a monoclonal or oligoclonal immune reponse to the underlying tumor.

Theoretically, a similar etiologic mechanism could be responsible for the M components in cold agglutinin disease, where, in some cases, the cold agglutinins have been shown to bind autologous and allogeneic lymphocytes,[11] presumably because lymphocytes bear surface antigens of the I/i antigen system.[18] Monoclonal cold agglutinins could therefore be produced by an autoreactive B cell clone in response to a lymphoid neoplasm expressing an epitope that is recognized by the cold antibody. Our data in patient 1, however, argue against this view. To address the question of cold autoantibody origin, the patient's chromosomally abnormal clone of B cells was immortalized and the culture supernatants contained a cold agglutinin with the same IgM, kappa isotype, and Pr specificity as the antibody present in the plasma of this patient. Thus, this proves that the cold autoantibody was derived from a chromosomally aberrant clone of lymphocytes.

The present findings support the view that chronic cold agglutinin disease may include a range of clonal disorders of increasing severity. For instance, a clone of B cells is present, but not detectable by laboratory methods such as those used in this study, and patients may or may not have an associated M component (patients 6 through 9); or, a clone of B cells has expanded and undergone additional somatic genetic change that is now recognized cytogenetically but evidence for lymphoma is lacking with standard diagnostic measures (patients 2 and 4). Finally, a clone of B cells has expanded to the point that it can be diagnosed as a lymphoma (patients 1 and 3).

The cytogenetic abnormality present in all four patients with a karyotypically abnormal clone included trisomy 3. It was the only abnormality in cases 2 and 3. Trisomy 3 is uncommon in human neoplasia, but has been reported occasionally in various B cell and T cell disorders, including multiple myeloma, angioimmunoblastic lymphadenopathy, chronic lymphocytic leukemia (CLL), and adult T-cell leukemia.[19-23] It almost always occurs in association with other cytogenetic alterations, suggesting that an extra copy of one or more genes on chromosome 3 confers only a slight additional growth advantage in a lymphoid clone. As with trisomy 12, which was observed in two of our cases and is common in B-CLL,[22] the specific genes involved in the pathogenesis of these low-grade lymphoproliferative disorders remain to be identified.

The cytogenetic findings in this study do provide, however, the first indication of an unusual nonrandom chromosomal change, trisomy 3, associated with cold agglutinin disease, as well as additional data on the clonal nature of this disorder. The results of this study also demonstrate that, in one patient, the defined B cell clone was the source of the monoclonal cold autoantibody.

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REFERENCES

1. Christenson WN, Dacie JV, Croucher BEE: Electrophoretic studies on sera containing high-titre cold haemagglutinins: Identification of the antibody as the cause of an abnormal γ 1 peak. Br J Haematol 3:262, 1957

2. Harboe M, Torsvik H: Protein abnormalities in cold haemagglutinin syndrome. Scand J Haemat 6:416, 1969

3. Petz LD, Branch DR: Serological tests for the diagnosis of immune hemolytic anemia, in McMillan R (ed): Methods in Hematology: Immune Cytopenias. New York, Churchill Livingstone, 1983

4. Mollison PL: Blood Transfusion in Clinical Medicine Techniques for detecting red cell antigens and antibodies. London, Blackwell Scientific Publications, 1983, p 552

5. Olson P, Weiblen BJ, O'Leary JJ, Moscowitz AJ, McCullough J: A simple technique for the inactivation of IgM antibodies using dithiothreitol. Vox Sang 30:149, 1976

6. Nowell P, Shankey TV, Finan J, Guerry D, Besa E: Proliferation, differentiation, and cytogenetics of chronic leukemic B lymphocytes cultured with mitomycin-treated normal cells. Blood 57:444, 1981

7. Miller G, Lipman M: Release of infectious Epstein-Barr Virus by transformed marmoset leukocytes. Proc Nat Acad Sci USA 70:190, 1973

8. Tsai C-M, Zopf DA, Yu RK, Wistar R, Jr, Ginsburg V: A Waldenstrom macroglobulin that is both a cold agglutining and a cryoglobulin because if binds N-acetylneuraminocyl residues. Proc Nat Acad Sci USA 74:4591, 1977

9. Parr DM, Connell GE, Powell AJ, Pruzanski W: A pathologic IgM occurring in serum as pentamer, dimer and monomer. J Immunol 113:2020, 1974

10. Reedman BM, Klein G: Cellular localization of an Epstein-Barr Virus (EBV)–associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. Int J Cancer 11:499, 1973

11. Crisp D, Pruzanski W: B-cell neoplasms with homogeneous cold-reacting antibodies (cold agglutinins). Amer J Med 72:915, 1982

12. Salmon SE: Immunoglobulin synthesis and tumor kinetics of multiple myeloma. Semin Hemat 10:135, 1973

13. Osserman EF, Takatsuki K: Plasma cell myeloma: Gamma globulin synthesis and structure. Medicine 42:357, 1963

14. Waldenstrom J: Clinical diagnosis and biochemical findings in material of 296 sera with M-type, narrow, γ globulins. Acta Med Scand Suppl 367:110, 1961

15. Latov N, Sherman WH, Nemni R, Galass J, Shyong JS, Penn AS, Chess L, Olarte MR, Rowland LP, Osserman EF: Plasma-cell dyscrasia and peripheral neuropathy with a monoclonal antibody to peripheral-nerve myelin. N Engl J Med 303:618, 1980

16. Intrator L, Andre C, Chenal C, Sultan C: A monoclonal macroglobulin with antinuclear activity. J Clin Path 32:450, 1979

17. Wagner O, Rasanen JA, Haltia K, Wasastjerna C: M components with antibody activity. Anti-smooth muscle, anti-thyroglobulin and anti-streptolysin-O activity in five M component sera. Ann Clin Res 3:86, 1971

18. Pruzanski W, Shumak KH: Biologic activity of cold-reacting antibodies (second of two parts). N Engl J Med 297:583, 1977

19. Lewis JP, Mackenzie MR: Nonrandom chromosomal aberrations associated with multiple myeloma. Hematological Oncology 2:307, 1984

20. Sanada I, Tanaka R, Kumagai E, Tsuda H, Nishimura H, Yamaguchi K, Kawano F, Fujiwara H, Takatsuki K: Chromosomal aberrations in adult T-cell leukemia: Relationship to the clinical severity. Blood 65:649, 1985

21. Godde-Salz E: Aberrations of chromosome 3. A marker of T-cell lymphomas? J Genet Hum 31:39, 1983

22. Han T, Sadamori N, Ozer H, Gajera R, Gomez GA, Henderson ES, Bhargava A, Fitzpatrick J, Minowada J, Bloom ML, Sandberg AA: Cytogenetic studies in 77 patients with chronic lymphocytic leukemia: Correlations with clinical, immunological, and phenotypic data. J Clin Oncol 2:1121, 1984

23. Kaneko Y, Larson RA, Variakojis D, Haren JM, Rowley J: Nonrandom chromosome abnormalities in angioimmunoblastic lymphadenopathy. Blood 60:877, 1982

Immunoglobulin Class Switch From IgG to IgA in a Patient With Smoldering Multiple Myeloma

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Serum of a 67-year-old male patient with smoldering multiple myeloma was shown to contain two monoclonal immunoglobulins, IgG and IgA. For the initial seven months, monoclonal IgG was predominantly elevated. During the next one year and eight months, however, serum concentration of the monoclonal IgA increased, with a concomitant decrease of IgG. *N*-terminal amino acid sequences of heavy and light chains separated from monoclonal IgG and IgA were analyzed. Both light chains were λ-type and showed identical amino acid sequences of variable regions. The heavy chains also had the same *N*-terminal amino acid sequence between IgG and IgA. These results strongly suggest that two monoclonal proteins, IgG and IgA, in this patient were produced by B lymphocytes within a clone and that class switch from IgG to IgA in immunoglobulin production during B cell differentiation has taken place in the clinical course of this case.

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IMMUNOGLOBULINS are composed of a pair of heavy (H-) and light (L-) polypeptide chains. Each chain consists of a variable (V-) region, which is an antigen-binding site, and a constant (C-) region. Immunoglobulin classes, such as IgM, IgD, IgG, and IgA, are defined by different C-regions of the H-chain (C_H) expressed together with the V-region of the H-chain (V_H).

Observing cells within the bursa of Fabricius in chickens, Cooper et al[1] found both IgM and IgG within individual bursa cells. Moreover, in vivo treatment of chickens and mice with antibodies to IgM significantly suppressed the development of IgM-, IgG-, and IgA-producing cells. This suppression experiment demonstrated that the expression of IgM synthesis was the primary event in B lymphocyte differentiation. The concept of an intraclonal class switch came from these observations.[1]

The class switch hypothesis was confirmed by genetic studies of Honjo and Kataoka.[2] They purified H-chain mRNAs from mouse myelomas of several classes for use as probes to measure the number of H-chain genes and showed deletion of C_H genes in myelomas that produce immunoglobulins of specific classes. An allelic deletion model for the mechanism of V_H-C_H recombination was proposed, and the C_H genes were supposed to be aligned on one chromosome in the order: V_H, spacer, μ, γ_3, γ_1, γ_{2b}, γ_{2a}, and α. In contrast, Migone et al[3] elucidated the gene sequence of human C_H by analyzing DNA of two healthy persons who were missing three and four immunoglobulin isotypes. Their findings demonstrated the order of human C_H genes to be μ, δ, γ_3, γ_1, ψ_ϵ, α_1, ψ_γ, γ_2, γ_4, ϵ, and α_2. These results suggest that an intraclonal class switch occurs in this order during human B lymphocyte differentiation.

We present the direct evidence of a class switch of monoclonal immunoglobulin from IgG to IgA during the clinical course of a patient with smoldering multiple myeloma.[4]

CASE REPORT

A 67-year-old male was noticed to have a bronchiectasia complicated with a monoclonal hypergammaglobulinemia in May 1975 at a local hospital. A single peak of serum monoclonal immunoglobulin was demonstrated on the γ-region of cellulose-acetate electrophoresis and IgG was over 3,000 mg/dL. In May 1977, IgG was 3,700 mg/dL and IgA was 1,900 mg/dL. The patient gradually developed dullness of the head and pain in distal parts of extremities and was admitted to the Niigata Shimin Hospital on Oct 7, 1977. Physical examination revealed the enlargement of several inguinal lymph nodes and rales in the lung fields. No hepatosplenomegaly was observed. The hemoglobin level was 8.5 g/dL, white blood cell counts 5,800/μL, and platelet counts 277 × 10^3/μL. Bone marrow aspiration revealed that plasma cells increased to 6.2% of nucleated cells. The total serum protein was 13.0 g/dL, and two separate peaks of monoclonal components were demonstrated first on the β-γ-region of cellulose-acetate electrophoresis. Serum IgG was 11,480 mg/dL, IgA 3,600 mg/dL, and IgM 92 mg/dL. Immunoelectrophoresis showed that two monoclonal components were IgG-λ and IgA-λ. Pyroglobulin was present in the serum.

Renal dysfunction was noticed, but there were no abnormal findings on a generalized bone survey. Histologic examination of the inguinal lymph node biopsy specimen showed diffuse infiltration of lymphocytes and plasma cells. The disease was diagnosed as multiple myeloma and treated with low doses of cyclophosphamide at first, because of hyperviscosity syndrome. However, leukocytopenia due to cyclophosphamide administration had developed, and then only plasmapheresis was performed. There was no sign of deterioration of multiple myeloma during the patient's course. He died of sepsis on Sept 19, 1979. On his admission, monoclonal IgG was remarkably greater than monoclonal IgA, but the reverse of two immunoglobulin values occurred in February 1978, and IgA increased gradually with concomitant decrease of IgG. IgG was 1,240 mg/dL and IgA was 10,080 mg/dL in April 1979 (Fig 1).

MATERIALS AND METHODS

Two monoclonal components in this patient, IgG and IgA, were isolated from the plasma of their predominant phase by a combination of ammonium sulfate precipitation (33% saturation), diethyl aminoethyl (DEAE)-cellulose (sodium phosphate buffer, pH 8.0), and DEAE-Sephadex (Tris-HCl buffer, pH 8.0) chromatography. The purity of isolated proteins was checked by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate[5] and double immunodiffusion with specific antisera.

Purified IgG (or IgA) (150 mg) was completely reduced and alkylated by the method of Waxdal and Konigsberg.[6] The H- and

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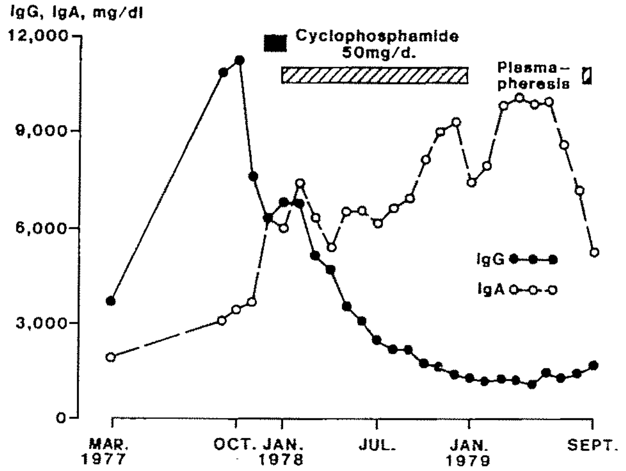

Fig 1. Clinical course of the patient.

L-chains were separated by gel filtration on Sephadex G-200 (2.5 × 95.0 cm) in 5 mol/L guanidine-HCl/1 mol/L acetic acid.

The *N*-terminal amino acid residue was determined by Edman's method.[7] Before the sequence analysis, H-chain peptides from IgG and IgA were digested with pyroglutamate aminopeptidase (from calf liver; Boehringer Manheim, Mannheim, FRG) by the method of Podell and Abraham.[8] Automated Edman degradation was carried out in a JEOL JAS-47K sequence analyzer (0.5mol/L Quadrol program; JEOL, Tokyo).[9] Phenylthiohydantoin derivatives were identified by thin-layer chromatography[10] and the back hydrolysis method.[11]

RESULTS

Amino-terminal sequence analysis. Both L-chains obtained from IgG and IgA, designated λ_γ- and λ_α-chains, respectively, had only tyrosine as the *N*-terminal residue. Because no amino acid was detected by the Edman degradation of both H-chains obtained from IgG and IgA, designated γ- and α-chains, respectively, these peptides were treated with pyroglutamate aminopeptidase to remove the *N*-terminal pyrrolidone carboxylic acid. Table 1 shows the results of the *N*-terminal amino acid sequence analyses. The *N*-terminal sequence of γ-chain was identical with that of α-chain. The sequences of λ_γ- and λ_α-chains were also identical.

When these *N*-terminal sequences were compared with other known sequences of the V-regions of H- and λ-chains, the V-regions of H- and λ-chains in this patient were found to belong to V_H III and V_λ III subgroups, respectively (the subgrouping is based on a classification by Kabat and Wu[12]).

DISCUSSION

Multiple myeloma is characterized by monoclonal immunoglobulin in the serum and osteolytic bone lesions associated with an increase of abnormal plasma cells in the bone marrow. Its course is generally progressive and fatal. Multiple myeloma whose course is not progressive and is stable for several years without chemotherapy was designated as smoldering multiple myeloma.[4] The present patient had remained stable for more than four years until he died in September 1979. Since October 1977 two remarkably elevated monoclonal proteins existed in the serum, but plasma cells in the bone marrow were less than 10% and bone lesion was not identified. Therefore, this patient was diagnosed as having smoldering multiple myeloma.

Multiple myeloma is usually associated with single monoclonal protein. Although the prevalence of double monoclonal components in the serum of patients with multiple myeloma is uncommon, it has been recognized in 1% of 6,141 recorded cases of multiple myeloma.[13]

Among 141 cases with double monoclonal proteins, frequencies of different associations were reported as follows: IgG and IgA, 32.6%; IgM and IgG, 24.1%; IgG and IgG, 17.0%; IgM and IgA, 8.5%; IgM and IgM, 7.8%.[14]

In 1970, Wang et al[15] first indicated that L-chains and *N*-terminal amino acid sequences of H-chains were identical between two monoclonal proteins (IgG_2-κ and IgM-κ) from a single patient. They speculated that during immunoglobulin synthesis different cells of a single clone synthesized immunoglobulins M and G and that the L-chains and the V_H of the two proteins were identical within the clone.

The idea of class switch in immunoglobulin production was brought up by Nossal et al[16] more than 20 years ago. They suggested that antibody-producing cells go through a sequence by which each synthesizes first 19S and later 7S antibody. This idea was developed and the concept of an intraclonal class switch was established by Cooper et al.[1] This was supported by the demonstration of coexistence of double monoclonal proteins originated from cells within a clone in patients with myeloma.[15] In some of the cases with IgG and IgA double monoclonal proteins, two monocomponents were demonstrated to be the products of common clonal cells by immunofluorescent study,[17] idiotypic antibody study,[18-20] both of them,[21,22] or analysis of amino acid sequences.[23-25] These findings suggested the possible sequence from IgG to IgA in a class switch. However, Gearhart et al,[26] observing a clone of cells developed from an antigen-stimulated mouse B lymphocyte, indicated two pathways to IgA production. One is a direct switch from IgM to IgA, and the other consists of two successive switches from

Table 1. N-terminal Amino Acid Sequences of H- and L-chains of IgG and IgA From the Patient

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
γ chain	PCA	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Val	Val	Lys	Pro	Gly							
α chain	PCA	Val	Gln	Leu	Val	Glu	Ser	Gly	Gly	Gly	Val	Val	Lys	Pro	Gly	Gly	Ser	Leu	Arg	Leu	Ser	Cys
λ_γ chain	Tyr	Glu	Leu	Thr	Gln	Pro	Pro	Ser	Val	Ser	Val	Ser	Ala	Gly	Gln	Thr	Ala	Thr	Ile	Thr	Cys	Ser
λ_α chain	Tyr	Glu	Leu	Thr	Gln	Pro	Pro	Ser														

PCA, pyrrolidone carbolic acid.

IgM to IgG and IgA during clonal expansion of antigen-stimulated B cells.

The concept of intraclonal class switch was perfectly compatible to an allelic deletion model for mouse V_H-C_H gene recombination.[2] Recently, the entire region of mouse C_H gene family was cloned by Shimizu et al,[27] the organization of which was shown as C_μ-C_δ-$C_{\gamma 3}$-$C_{\gamma 1}$-$C_{\gamma 2b}$-$C_{\gamma 2a}$-C_ϵ-C_α. As to the human study, C_H genes were identified on chromosome 14q32 in the order of μ, δ, γ_3, γ_1, ψ_ϵ, α_1, ψ_γ, γ_2, γ_4, ϵ, and α_2.[3] These results indicate that during differentiation of a single B lymphocyte, a given V_H gene is first expressed in combination with the $C\mu$ gene and then expressed with a different C_H gene in the order described earlier.

The patient described here was observed to have monoclonal protein of IgG more than two years before admission to the hospital and then showed remarkable double monoclonal immunoglobulins on admission. They were identified as IgG_λ and IgA_λ by immunoelectrophoresis. IgG was extremely more predominant than IgA in October 1977. However, the IgG value declined progressively during his clinical course. In contrast, IgA value kept increasing through his terminal stage, with crossing IgG in February 1978. The amino acid sequence analysis of variable regions in H- and L-chains from the patient's monoclonal IgG and IgA has been performed. The *N*-terminal amino acid sequence of γ-chain of IgG predominant phase was identical with that of α-chain of IgA predominant phase. In addition, the *N*-terminal sequences of λ_γ- and λ_α-chains were also identical. These results suggest that the variable regions of H-chains and the L-chains of IgG and IgA, respectively, are identical, which gives us a strong evidence that IgG and IgA were derived from a common clonal immunoglobulin-producing cell lineage. These results, together with the clinical finding that IgA in the patient plasma increased with a concomitant decrease of IgG, give enough evidence to conclude that neoplastic B lymphocytes within a clone in this case have undergone a class switch in C_H gene expression from γ to α with cell differentiation in vivo. This is a novel case of myeloma in which an immunoglobulin class switch from IgG to IgA was observed during the clinical course.

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REFERENCES

1. Cooper MD, Lawton AR, Kincade PW: A two-stage model for development of antibody-producing cells. Clin Exp Immunol 11:143, 1972

2. Honjo T, Kataoka T: Organization of immunoglobulin heavy chain genes and allelic deletion model. Proc Natl Acad Sci USA 75:2140, 1978

3. Migone N, Oliviero S, de Lange G, Delacroix DL, Boschis D, Altruda F, Silengo L, DeMarchi M, Carbonara AO: Multiple gene deletions within the human immunoglobulin heavy-chain cluster. Proc Natl Acad Sci USA 81:5811, 1984

4. Kyle RA, Greipp PR: Smoldering multiple myeloma. N Engl J Med 302:1347, 1980

5. Weber K, Osborn M: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406, 1969

6. Waxdal MJ, Konigsberg WH: The covalent structure of a human γG immunoglobulin. II. Isolation and characterization of the cyanogen bromide fragments. Biochemistry 7:1959, 1968

7. Iwanaga S, Wallen P: On the primary structure of human fibrinogen. Isolation and characterization of N-terminal fragments from plasmic digests. Eur J Biochem 8:189, 1969

8. Podell DN, Abraham GN: A technique for the removal of pyroglutamic acid from the amino terminus or protein using calf liver. Biochem Biophys Res Commun 81:176, 1978

9. Edman P, Begg G: A protein sequenator. Eur J Biochem 1:80, 1967

10. Bridgen J, Graffeo AP: Thin layer chromatography, in Perham RN (ed): Instrumentation in amino acid sequence analysis. London, Academic Press, 1975, p 117

11. Mendez E, Lai CY: Regeneration of amino acids from thiazolinones formed in the Edman degradations. Anal Biochem 68:47, 1975

12. Kabat EA, Wu TT: Sequences of immunoglobulin chains. Bethesda Md, NIH publication No. 80-2008, 1979, p 24

13. Oriol R, Huerta J, Bouvet JP, Liacopoulos P: Two myeloma globulins, IgG_1-κ and IgG_1-λ, from a single patient (Im). Immunology 27:1081, 1974

14. Bouvet JP, Feingold J, Oriol R, Liacopoulos P: Statistical study of double paraproteinemias. Evidence for preferential association of same light chain types. Biomedicine 22:517, 1975

15. Wang AC, Wilson SK, Hopper JE, Fudenberg HH, Nisenoff A: Evidence for control of synthesis of variable regions of the heavy chains of immunoglobulins G and M by the same gene. Proc Natl Acad Sci USA 66:337, 1970

16. Nossal GJV, Szenberg A, Ada GL, Austin CM: Single cell studies on 19S antibody production. J Exp Med 119:485, 1964

17. Costea N, Yakulis VJ, Libnoch JA, Pilz GG, Heller P: Two myeloma globulins (IgG and IgA) in one subject and one cell line. Am J Med 42:630, 1967

18. Fair DS, Drueger RG, Eleich GJ, Kyle RA: Studies on IgA and IgG monoclonal proteins derived from a single patient. I. Evidence for shared individually specific antigenic determinants. J Immunol 112:201, 1974

19. Grubb AO, Zettervall OH: Immunochemical evidence for a common variable region in three immunoglobulin classes in the same individual. Proc Natl Acad Sci USA 72:4115, 1975

20. Fair DS, Schaffer S, Krueger RG: Development of monoclonal IgA and an apparent IgG in a patient with macroglobulinemia: Sharing of individually specific antigenic determinants among IgM, IgA and IgG. J Immunol 117:944, 1976

21. Rudders RA, Yukulis V, Heller P: Double myeloma. Production of both IgG type lambda and IgA type lambda myeloma proteins by a single plasma cell line. Am J Med 55:215, 1973

22. Rudders RA, Ross R: Partial characterization of the shift from IgG to IgA synthesis in the clonal differentiation of human leukemic bone marrow-derived lymphocytes. J Exp Med 142:549, 1975

23. Wolfenstein-Todel C, Franklin EC, Rudders RA: Similarities of the light chains and the variable regions of the heavy chains of the IgG_2-λ and IgA_1-λ myeloma proteins from a single patient. J Immunol 112:871, 1974

24. Fair DS, Sledge C, Krueger RG, Mann KG, Hood LE: Studies on IgA and IgG monoclonal proteins derived from a single

patient. Evidence for identical light chains and variable regions of the heavy chain. Biochemistry 14:5561, 1975

25. Sledge C, Fair DS, Black B, Krueger G, Hood L: Antibody differentiation: Apparent sequence identity between variable regions shared by IgA and IgG immunoglobulins. Proc Natl Acad Sci USA 73:923, 1976

26. Gearhart PJ, Hurwitz JL, Cebra JJ: Successive switching of antibody isotypes expressed within the line of a B-cell clone. Proc Natl Acad Sci USA 77:5424, 1980

27. Shimizu A, Takahashi N, Yaoita Y, Honjo T: Organization of the constant-region gene family of the mouse immunoglobulin heavy chain. Cell 28:499, 1982

Induction of Tac Antigen and Proliferation of Myeloid Leukemic Cells by ATL-Derived Factor: Comparison With Other Agents That Promote Differentiation of Human Myeloid or Monocytic Leukemic Cells

By Seiko Yamamoto, Toshio Hattori, Masao Matsuoka, Toshinori Ishii, Norio Asou, Masafumi Okada,
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We studied seven cases of myeloid leukemia at various differentiation stages to investigate the response of leukemic cells to phorbol 12-myristate 13-acetate (PMA) and various biological factors. γ-Interferon (γ-IFN)-treated cells expressed higher amounts of Fc receptors on leukemic cells in five out of seven cases. Expression of HLA-DR antigen of γ-IFN-treated leukemic cells was significantly enhanced in three cases. PMA did not induce Fc receptors or HLA-DR antigen on these cells. Induction of Tac antigen, a putative interleukin 2 (IL 2) receptor, was observed in two cases after cultivation with PMA or with a novel lymphokine, adult T cell leukemia-derived factor (ADF). Cells from one of these patients expressed Tac antigen immediately after cell separation, and expression of Tac antigen was augmented by PMA and ADF. Interleukin 1 (IL 1) or IL 2 did not induce Tac antigen. Leukemic cells from this patient also proliferated vigorously in the presence of ADF but not PMA, IL 1, or IL 2.

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MYELOID LEUKEMIC cells have been classified according to the French-American-British (FAB) classification[1,2] based on their morphological and histochemical characterizations. The biology of myeloid leukemic cells has been poorly understood as compared with lymphoid malignancies. Recently, characterization of their surface markers using monoclonal antibodies (mAb) was begun, but the antigens defined by these mAb have not been well characterized. Although leukemic cells have been considered to be frozen at various differentiation stages, some fresh leukemic cells and leukemic cell line cells differentiate to maturer cells under appropriate stimuli. Interferon (IFN) increases the expression of Fc receptors of human phagocytes,[3] promotes differentiation of promyelocytic leukemia cell line cells (HL60),[4,5] and induces antibody-dependent cellular cytotoxicity by histiocytic lymphoma cell line cells (U937).[6] Some fresh myeloid leukemic cells are also enhanced in the expression of monocyte-related antigens or the amounts of Fc receptors after treatment with γ-IFN.[4,7] Phorbol 12-myristate 13-acetate (PMA) also promotes differentiation of myeloid leukemic cells and monocytic leukemia cell line cells and increases their functional activities.[6,8]

Adult T cell leukemia (ATL) is a distinct clinical entity.[9,10] Surface phenotypic analyses of ATL cells show that they are derived from activated T cells,[11] and production of a number of lymphokines from ATL-derived cell line cells has been described.[12] Miyoshi et al[13] also reported extramedullary granulopoiesis in hamsters in which human T cell lymphotropic virus type I (HTLV-I)-infected cell line cells, MT-1, were transplanted. Recently, adult T cell leukemia-derived factor (ADF), a novel lymphokine that was produced by another ATL-derived cell line, ATL-2, was described by Yodoi et al.[14-17] ADF is a unique lymphokine that induces the expression of Tac antigen,[18,19] a putative interleukin 2 (IL 2) receptor, on a human natural killer cell line, YT. ADF also enhances the expression of Tac antigen on HTLV-I-infected cell line cells and may play an important role in their proliferation,[17] but its biological significance and effects on fresh leukemic cells are unknown.

Here we have examined the effects of various agents on surface phenotypic and morphological changes of myeloid leukemic cells. The effects of other lymphokines, including interleukin 1 (IL 1) and IL 2, have also been studied in selected cases.

MATERIALS AND METHODS

Patients. All patients were admitted to Kumamoto University Hospital and were diagnosed according to the FAB classification. Patient profiles are listed in Table 1.

Reagents. PMA was from Consolidated Midland (Brewster, NY). Recombinant γ-IFN and recombinant IL 2 were kindly provided by Kyowa Hakko Co (Tokyo) and Takeda Pharmaceutical Co (Osaka, Japan), respectively. Partially purified human IL 1 was kindly provided by Dr J.J. Oppenheim (National Cancer Institute, Bethesda, Md). Fetal calf serum (FCS) was from Flow Laboratories (North Ryde, New South Wales, Australia). RPMI 1640 medium was from Nissui (Tokyo). Bovine serum albumin (BSA) was from Sigma Chemical Co (St Louis). Fluorescein isothiocyanate (FITC) was obtained from BBL Microbiology Systems (Cockeysville, Md). FITC-labeled OKT3 (pan T), T11 (E rosette receptor), Ia2 (HLA-DR) were from Ortho Pharmaceutical Co (Raritan, NJ), and FITC-unlabeled anti-J5 (common acute lymphocytic leukemia antigen) and FITC-labeled Mo1 (monocytes and granulocytes) were from Coulter Diagnostics (Hialeah, Fla). FITC-labeled Leu-12 mAb, which detects B cell lineage cells, was from Becton Dickinson Monoclonal Center (Mountain View, Calif). Anti-Tac mAb was kindly provided by Dr T. Uchiyama at Kyoto University. FITC-labeled goat $F(ab')_2$ fragments of antimouse IgG was from Tago, Inc (Burlingame, Calif). Sephadex G25 and protein A-Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). Terminal deoxytransferase (TdT) activity of leukemic cells was examined using a kit from BRL (Gaithersburg, Md).

Culture conditions. Leukemic cells were separated from bone marrow taken from JM and FF and from heparinized peripheral blood in other cases, using Ficoll-Conray density gradient centrifugation. Separated cells were washed three times with RPMI 1640 medium and were cultured at 37 °C with or without stimulating

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Table 1. Clinical Profiles and Hematologic Data of Patients

Patient	Age	Sex	WBC (10^9/L)	Hemoglobin (g/dL)	Platelets (10^9/L)	Hepatomegaly	Splenomegaly	Diagnosis
JM	57	M	1.0	10.8	94	—	—	M1
FF	59	M	2.5	6.0	24	—	—	M1
FI	60	F	52.4	6.7	407	—	—	M1
YN	53	F	14.2	5.6	15	—	—	M2
TT	43	M	21.8	12.0	223	—	±*	M4
SM	84	F	215.6	13.2	192	—	—	M5a
KS	76	F	10.5	6.5	20	—	±	M5b

*Less than twofold increase in size, examined by abdominal echography.

agents in RPMI 1640 medium containing streptomycin (100 μg/mL), penicillin G (100 U/mL), and 10% FCS (complete medium) at a cell density of 1×10^6/mL in a total volume of 2 mL in 24-well tissue culture plates (Linbro, Flow Labs, McLean, Va) in a humid atmosphere with 5% CO_2. After cultivation, cells were harvested by washing wells vigorously with phosphate-buffered saline (pH 7.4) containing 0.5% BSA and 0.1% sodium azide (PBS–BSA) using Pasteur pipettes. After two washes with the same buffer, surface markers were examined. For morphological examination, cells (1×10^5) were spun down onto glass slides using a Cytospin 2 (Shandon Southern Products, Cheshire, England) and stained with May-Grünwald-Giemsa. Viabilities of cells were examined under a microscope by trypan blue dye exclusion and always exceeded 90%.

Purification of ADF. Human cell line ATL-2, established from an acute ATL patient,[20] was cultured in serum-free RPMI 1640 medium and incubated for four days. The cell-free culture supernatant (2 L) was concentrated at 10 mL using Amicon's DC-2 hollow fiber system (H1P10-8 hollow fiber, molecular cut at 10 kd; Amicon Corp, Lexington, La). The concentrated supernatant was separated by Sephacryl S-200 gel filtration, followed by stepwise elution in sodium phosphate buffer (10 mmol/L to 0.5 mol/L, pH 6.8) by hydroxylapatite column chromatography using a Beckman high-pressure liquid chromatography (HPLC) system (Berkeley, Calif). ADF activity was eluted at 100 mmol/L of the buffer. After concentrating active fractions, ADF was purified by HPLC reverse-phase column chromatography (Synchrom, Lindon, Ind). The reverse-phase chromatography gave an ADF activity peak corresponding to about 12-kd protein with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. ADF activity was measured by induction of Tac antigen on YT cells, as described previously.[15] One unit of ADF induces 50% of maximum increase of Tac antigen expression on YT cells.

Assay of Fc receptors. Human IgG1 was obtained from the serum of a patient with multiple myeloma by diethylaminoethyl (DEAE)-cellulose chromatography, followed by affinity chromatography using protein A-Sepharose. Five milliliters of purified IgG1 (10 mg/mL) was incubated with 1 mg of FITC in 0.5 mol/L carbonate buffer (pH 9.5) according to Goldstein et al,[21] and labeled protein and free FITC were separated by Sephadex G25. F-P ratio of FITC–IgG1 was 1.4. Usually, 3 μg of FITC–IgG1 was added to 2×10^5 cells in 150 μL of PBS–BSA. After incubation for 30 minutes on ice, cells were washed twice with buffer, and FITC-positive cells were determined by a laser flow cytometry system (Spectrum III; Ortho Diagnostic Systems, Westwood, Mass) by exposure to a light of 480 nm at an intensity of 20 mW. The cutoff point that gave less than 1% positive cells was determined for each cell when 100-fold excess of unlabeled IgG1 was added. Results were expressed as mean fluorescence intensity (MFI).

Cell surface markers and anti-Tac immunoprecipitation. Cell surface markers were determined by direct or indirect immunofluorescence methods. To block the binding of Fc receptors, cells were incubated with an optimal amount of mAbs in the presence of 50 μL of normal AB serum on ice. For the indirect immunofluorescence assay, cells were further incubated with 50 μL of FITC-labeled goat $F(ab')_2$ antimouse IgG (1:160 dilution) for another 30 minutes on ice. Cells were analyzed by laser flow cytometry as described.

The Tac antigen immunoprecipitated by anti-Tac antibody from labeled cell lysates was analyzed by SDS-PAGE, as described elsewhere.[22] As a positive control, normal peripheral blood lymphocytes, cultured for three days in the presence of 2 μg/mL concanavalin A (Con A) (Uppsala, Sweden), were used. Briefly, 5×10^7 cells were surface labeled with $Na^{125}I$ (New England Nuclear, Boston) by the lactoperoxidase method and were washed and lysed in extraction buffer (0.5% Nonidet P-40, 10 mmol/L Tris-HCl, 0.15 mol/L NaCl, 1 mmol/L phenylmethylsulfonylfluoride, pH 7.2). For immunoprecipitation, aliquots of the labeled cell lysates were incubated overnight at 4 °C with SPA-coupled Sepharose 4B and 5 μg of anti-Tac (partially purified by SPA-coupled Sepharose 4B column from hybridoma ascites fluid). The beads were serially washed with the buffer. The immunoprecipitates were analyzed by SDS-PAGE on 10% polyacrylamide gels and visualized by autoradiography.

Proliferative assays of leukemic cells. Fifty thousand leukemic cells in 200 μL of medium were cultured for 72 hours in the presence of various agents in flat-bottomed plates (Falcon microtest plates III; Falcon, Oxnard, Calif). The cultured cells were pulsed with ^3H-thymidine (2 Ci/mol, Amersham Corp, Arlington Heights, Ill) for the last 18 hours of culture, followed by precipitation onto glass fiber filters. Radioactivity was countered by a liquid scintillator.

Binding assay by ^{125}I-labeled IL 2. Recombinant IL 2 was labeled with ^{125}I using Bolton Hunter's reagent (New England Nuclear). The specific activity of the IL 2 was 7,000 cpm/ng, and the bindability was examined with an increasing number of ATL-2 cells at a constant input of the radiolabeled reagent. More than 80% of radiolabeled reagent was bindable to ATL-2. One million cells were suspended in 100 μL of complete medium in 1.5-mL Eppendorf micro test tubes. One hundred microliters of complete medium containing an appropriate amount of ^{125}I-IL 2 was added to the cell suspension. These tubes were incubated at 37 °C for 30 minutes. The mixture was centrifuged at 3,000 rpm for two minutes through a 200-μL layer of mixture of 20% olive oil and 80% Di-*n*-butyl-Phthalate (Nakarai Co, Kyoto, Japan) to remove residual radioactivity in 400-μL Eppendorf micro test tubes. The tip of the tube was cut and the radioactivity bound to the cells was counted by a gamma counter. The supernatant was also counted as free radioactivity. As a negative control, a cold inhibition assay with 500-fold excess of IL 2 was carried out. Specific bound radioactivity was calculated by subtracting the cold inhibition count, as described previously.[16]

RESULTS

Characterization of leukemic cells. Cell surface markers of leukemic cells immediately after cell separation are listed in Table 2. Relative proportions of leukemic cells in all cases exceeded 80% in cytocentrifuge preparations stained

Table 2. Surface Phenotypic Characterization of Leukemic Cells

Patient	TdT	Mol (%)	OKT11 (%)	Leu-12 (%)	OKIa-2 (%)	J5 (%)	FcR (MFI)	Tac (%)
JM	–	NT	2.7	2.2	80.5	1.2	19.1	1.8
FF	–	58.6	4.4	2.6	74.9	1.5	20.3	1.5
FI	–	70.8	0.9	2.0	54.7	0.6	41.4	7.4
YN	–	49.6	5.2	4.3	73.1	1.0	37.0	1.5
TT	–	96.7	3.1	2.8	66.3	2.1	42.5	1.0
SM	–	95.9	1.0	3.9	3.6	1.0	76.3	0.7
KS	–	NT	2.5	3.5	88.4	1.5	85.2	1.2

FcR, Fc-receptor; MFI, mean fluorescence intensity; NT, not tested; –, negative.

by May-Grünwald-Giemsa. Residual normal T or B cells defined by OKT11 or Leu-12 were less than 6% in all cases. All cells were negative for TdT and J5. Mol antigen was detected in all cases examined. The amounts of Fc receptors were high in SM and KS, which belong to M5. HLA-DR antigen was detected in all cases except in SM. A low percentage of cells in FI were positive for anti-Tac mAb.

Fc receptors and HLA-DR antigen of cultured cells. Fc receptors and HLA-DR antigen of cultured cells (72 hours) were observed. Cells were cultured with γ-IFN (300 U/mL; unit used here is International Unit) or PMA (10^{-9} mol/L). PMA, which is well known to promote differentiation of myeloid leukemic cells to macrophage-like cells,[8] neither induced the HLA-DR antigen nor increased significantly the amounts of Fc receptors (Fig 1).

γ-IFN–treated cells expressed HLA-DR antigen in increased numbers over medium-treated cells from FF, TT, and SM. Subtle changes were observed in cells from FI, YN, and KS. HLA-DR antigen could not be induced when cells from JM were used.

The amounts of Fc receptors of cells cultured with medium only increased over those of fresh isolated cells in TT, KS, and SM that belonged to M4 and M5 stages. Cells that responded to γ-IFN with respect to HLA-DR antigen expression also showed higher amounts of Fc receptors after treatment with γ-IFN.

Expression of Tac antigen of cultured cells. Tac antigen was detected on cells from FI that were cultured with medium only (Fig 1). A high percentage (77.4%) of PMA-treated FI cells also expressed Tac antigen. These increases of Tac antigen-positive cells could not be attributed to either the proliferation of residual T or B cells or the selective proliferation of Tac antigen-positive cells because 18% of PMA-treated cells were positive for OKT11 and 4.2% were positive for Leu-12. Also, a threefold increase of Tac antigen-positive cells was observed as early as six hours after cultivation with or without PMA. These increases of Tac antigen-positive cells were maintained only in the presence of PMA, and Tac antigen-positive cells of medium-cultured cells gradually decreased day by day (Fig 2). Tac antigen was also induced by PMA when cells from FF were used. However, materials were available only from bone marrow, so only restricted studies have been done in FF.

Effects of ADF (10 U/mL) on Tac antigen expression on leukemic cells were examined in four cases. Cells from FF and FI expressed Tac antigen after cultivation with ADF (Table 3). ADF increased Tac antigen-positive cells in a dose-dependent manner. IL 1 and IL 2 did not induce any change of Tac antigen-positive cells in case FI (Fig 3).

Expression of Tac antigen and Fc receptors on FI cells cultured with various agents expressed by histogram of flow cytometric analyses were summarized in Fig 4. γ-IFN–treated cells did not induce Tac antigen in all cases examined.

The immunoprecipitates of Tac antigen on FI leukemic

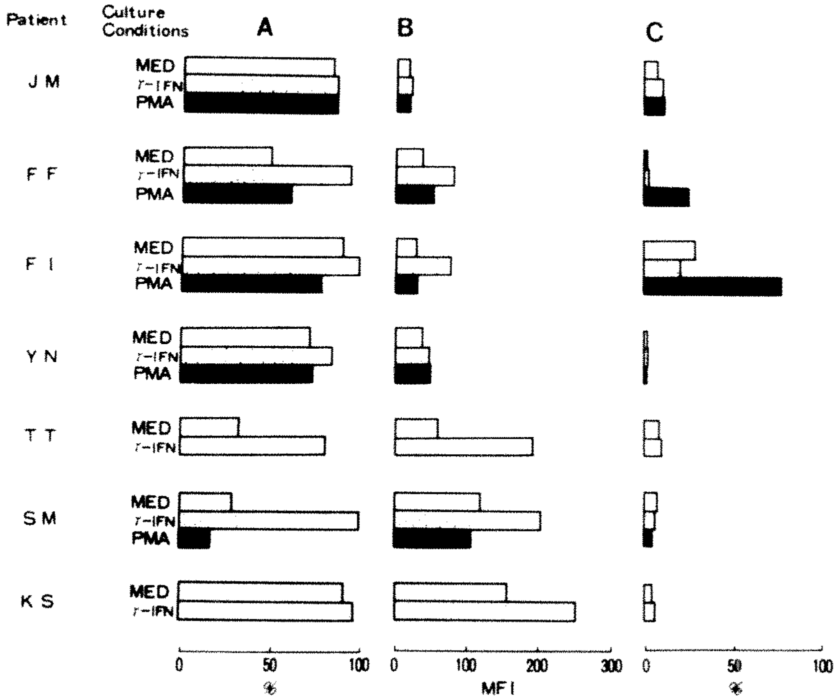

Fig 1. Expression of (A) HLA-DR antigen, (B) Fc receptors, and (C) Tac antigen on cultured leukemic cells from seven patients at day 3. Cells were cultured with γ-IFN (300 U/mL), PMA (10^{-9} mol/L), or medium only.

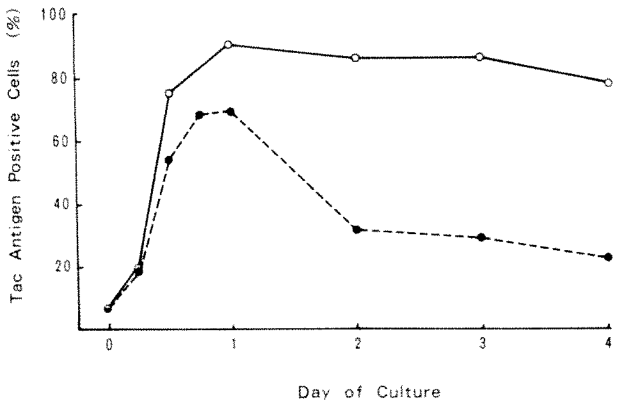

Fig 2. Time course of Tac antigen expression. Cells from FI were cultured for up to four days in culture medium alone (●) or culture medium containing PMA (10^{-9} mol/L) (○).

cells that were cultured with ADF for three days were analyzed by SDS-PAGE (Fig 5). For reference, the 60- to 65-kd Tac protein immunoprecipitated from Con A-activated normal T lymphocytes is shown (lane a). Tac antigen identified on ADF-treated FI leukemic cells migrated at the same point as that of Con A-activated normal T cells (lane b).

Morphological changes of leukemic cells. Twenty percent of FI cells cultured with medium alone showed azurophilic granules in the cytoplasm that were not seen when freshly isolated cells were examined. PMA-treated FI cells showed increased membrane ruffling and vacuoles in the cytoplasm, and azurophilic granules were also seen. FI cells cultured with γ-IFN showed a slightly lowered nuclear-cytoplasm ratio and a subtle increase of azurophilic granules. ADF-treated cells showed abundant amounts of azurophilic granules in their cytoplasm, like promyelocytes (Fig 6). However, mature neutrophils could not be observed even after 14 days culture in the presence of ADF. We have also examined the morphology of PMA- or γ-IFN–treated cells from other cases. Cells from TT, KS, SM, and FF showed low nuclear-cytoplasm ratios when cultured with γ-IFN, and increased membrane ruffling and vacuoles were seen when PMA was used. Cells from JM and YN did not respond to γ-IFN morphologically; however, PMA-treated cells showed increased membrane ruffling, but these changes were less significant as compared with other cases.

Proliferative responses of leukemic cells. ^{3}H-thymidine uptake of leukemic cells from FF, YN, and SM was measured in the presence of ADF (1 to 30 U/mL). No proliferative responses were observed. Vigorous proliferation of FI cells occurred in a dose-dependent manner when ADF was added. IL 1 and IL 2 did not induce any proliferation (Fig 7). IL 2 at a concentration of 10 to 100 U/mL did not promote proliferation of FI cells. PMA (10^{-9} mol/L) did not induce proliferation of FI cells, and a synergistic effect of ADF and IL 2 on proliferation of FI cells could not be observed (data not shown).

Table 3. Induction of Tac Antigen by ADF on Fresh Leukemic Cells From Four Patients

Patient	FF (%)	FI (%)	YN (%)	SM (%)
Medium	1.0	29.5	1.2	6.8
ADF (10 U/mL)	24.8	77.4	2.7	8.4

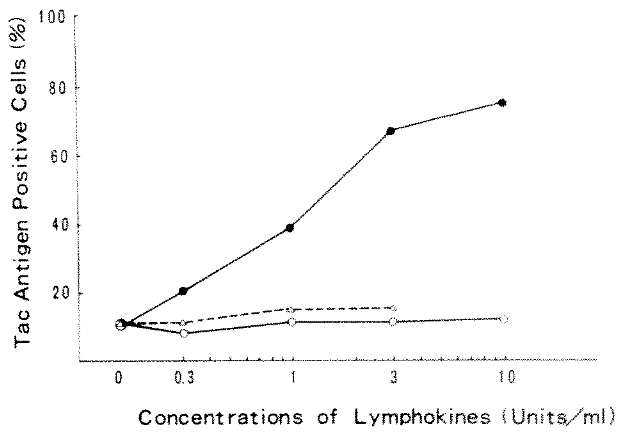

Fig 3. Dose-response curves for Tac antigen expression. Cells from FI were cultured with ADF (●), IL 1 (△), or IL 2 (○) for three days.

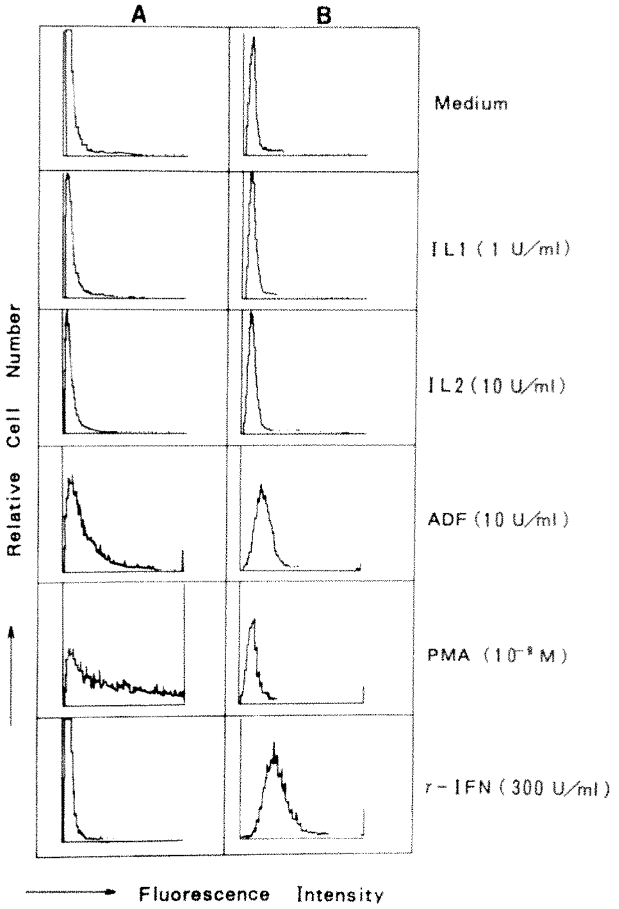

Fig 4. Histogram of flow cytometric analyses of (A) Tac antigen and (B) Fc receptor expression on cultured cells from FI with or without various agents at day 3.

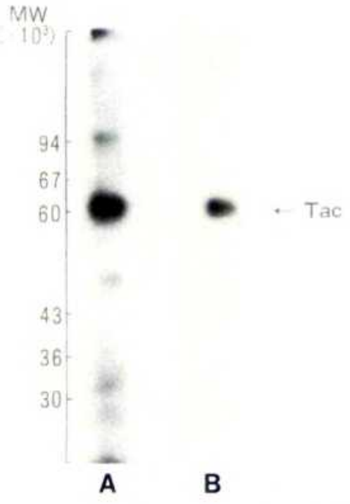

Fig 5. Autoradiography of anti-Tac immunoprecipitation of ADF-treated FI leukemic cells (lane B). For reference, Tac antigen on normal Con A blasts is shown (lane A).

Binding assay by ^{125}I-IL 2. The binding assay with ^{125}I-IL 2 showed that fresh leukemic cells had fewer than 200 total IL 2 receptor sites per cell. Because of the low net count of specifically bound ^{125}IL 2, the analysis of IL 2 receptor sites by the method of Scatchard analysis was difficult. When the cells were treated with ADF for 24 hours, a dose-dependent increase in the number of IL 2 receptor sites was observed (Table 4). ADF (10 U/mL) induced about 100 high-affinity IL 2 receptor sites (kd; 200 pmol/L) and 1,300 low-affinity IL 2 receptors (kd; 9 nmol/L). The high-affinity receptor sites (kd; 380 pmol/L) increased to 500 per cell, while the low-affinity receptor (kd; 8 nmol/L) sites increased to 2,000 in the presence of 100 U/mL of ADF.

DISCUSSION

We studied seven cases of myeloid leukemia at various differentiation stages. The amounts of Fc receptors increased by cultivation of cells at stage M4 or M5, suggesting that cytophilic IgG is binding to freshly isolated leukemic cells in vivo. In fact, Fc receptors on these freshly isolated leukemic cells increased by 20% to 30% after six times' washing (data not shown). The amounts of Fc receptors correlated well with the FAB classification; cells from M4 and M5 had greater amounts of Fc receptors than did cells from M1 and M2.

γ-IFN–treated cells expressed greater amounts of Fc receptors on all leukemic cells that belonged to stage M4 or M5. Fc receptors of leukemic cells from two out of four other cases also increased after treatment with γ-IFN.

HLA-DR antigen defined by OKIa2 mAb was detected in all cases except SM when examined immediately after cell separation. HLA-DR antigen was detected in medium-cultured cells from SM. Significant increases of HLA-DR antigen-positive cells by γ-IFN treatment were observed in FF, TT, and SM in which low percentages of leukemic cells expressed HLA-DR antigen. These increases could not be attributed to Fc receptor-mediated binding because anti-Tac mAb did not bind to these cells and Fc receptors were blocked by AB human serum. Subtle increases of HLA-DR

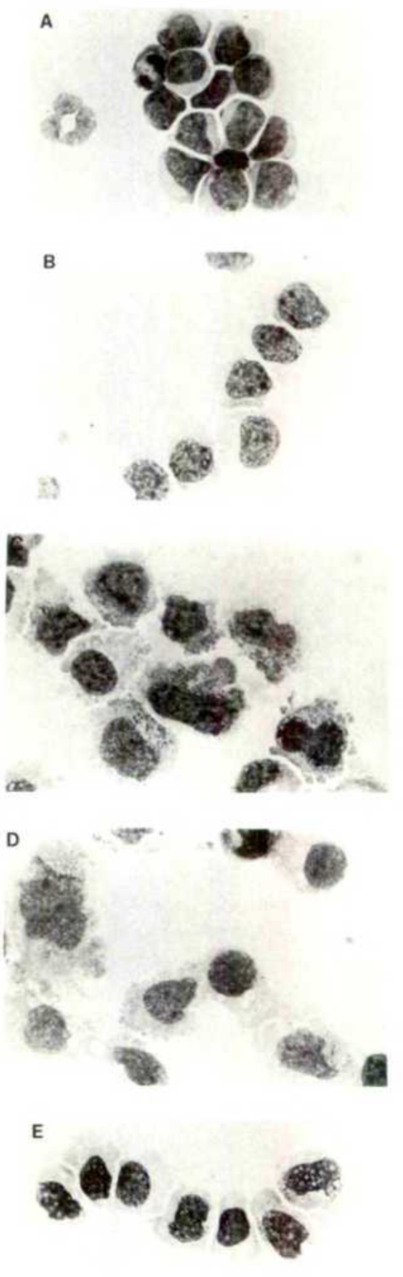

Fig 6. Morphology of fresh and three-days'-cultured FI leukemic cells. Cytocentrifuge preparation of leukemic cells were stained with May-Grünwald-Giemsa staining. (A) Fresh leukemic cells. (B) Cultured with medium only. (C) Cultured with ADF (10 U/mL). (D) Cultured with PMA (10^{-9} mol/L). (E) Cultured with γ-IFN (300 U/mL). (Original magnification ×1,000; current magnification ×400).

antigen-positive cells were observed in three other cases but were not significant. The effects of γ-IFN on leukemic cells were more prominent on Fc receptors than on HLA-DR antigen expression. We could not induce Tac antigen on γ-IFN–treated leukemic cells in all cases, although induction of Tac antigen on human leukemic and normal monocytic cells by γ-IFN was recently reported.[23] The reasons for the

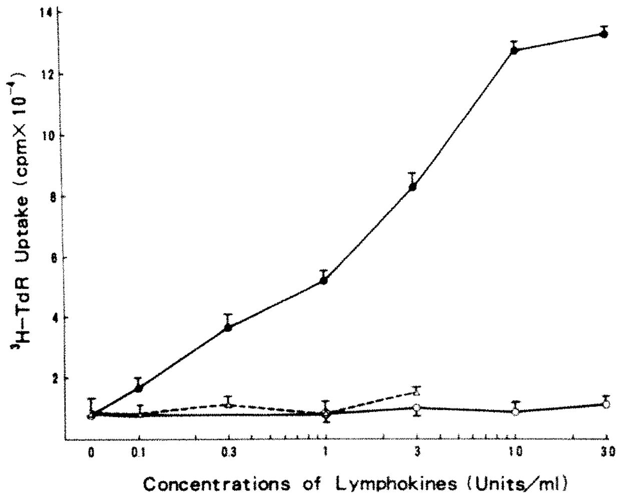

Fig 7. Dose-response curves for ^{3}H-thymidine uptake. Cells were cultured with ADF (●), IL 1 (△), or IL 2 (○), and ^{3}H-thymidine uptake was measured after an 18 hours' pulse after 72 hours cultivation.

discrepancies between our results and theirs were not clear. Analysis of messenger RNA of IL 2 receptor gene of γ-IFN-treated monocytic cell line cells would answer the question.

PMA, which is known to differentiate HL60 cells to macrophage-like cells, did not induce Fc receptors or HLA-DR antigen. These findings suggest that the mechanisms that promote differentiation of HL60 cells may be different, although both γ-IFN and PMA promote differentiation of HL60 cells to macrophage-like cells.

Unexpected findings during these studies were the detection of Tac antigen on leukemic cells from two cases (FF and FI). These leukemic cells did not carry lymphoid markers, such as TdT, J5, T3, T11, and Leu-12, and expressed Mo1 antigen, confirming their myeloid origin. Karyotypic analyses of these two leukemic cells did not demonstrate Philadelphia chromosomes. These leukemic cells belong to M1 stage according to the FAB classifications. Electron-microscopic observations also confirmed their myeloid origin (unpublished observations, July 1985).

Expression of Tac antigen on FI and FF leukemic cells was greatly enhanced in the presence of PMA or ADF. Tac antigen on FI leukemic cells cultured with ADF was analyzed on one-dimensional SDS-PAGE. No apparent difference between molecular weight of Tac antigen induced on FI cells and normal Con A blasts was detected. Because Tac antigen is known to recognize IL 2 receptors,[19] a binding assay using ^{125}I-IL 2 was done. Binding of ^{125}I-IL 2 increased when FI cells were treated with ADF, although the numbers of receptor sites was low, compared with those on ATL cells, which express from 960 to 3,300 high-affinity and from 37,500 to 330,000 low-affinity receptors per cell.[24] FI leukemic cells proliferated well in the presence of ADF in a dose-dependent manner, although PMA, IL 1, and IL 2 did not induce the proliferation of FI cells. Unresponsiveness to IL 2 despite the presence of high- and low-affinity IL 2 receptors is also known when ATL cells were used,[24] and the biological significance of the presence of Tac antigen on these leukemic cells has not yet been clarified. Furthermore, ADF-treated cells had abundant azurophilic granules in their cytoplasm, compared with medium-cultured cells, suggesting that responding leukemic cells differentiate to promyelocytes. The proliferation of FF cells was not induced by ADF. We could not explain the differences between leukemic cells from these two patients. However, Tac antigen was expressed only in the presence of PMA or ADF, and the relative proportions of Tac antigen-positive cells were comparatively low in FI.

Table 4. Binding of ^{125}I-Labeled IL 2 to ADF-Treated FI Leukemic Cells

	High Affinity		Low Affinity	
	No. of Receptors per Cell	kd (nmol/L)	No. of Receptors per Cell	kd (nmol/L)
Medium	NC		NC	
ADF (10 U/mL)	120	0.20	1,300	9.0
ADF (100 U/mL)	500	0.38	2,000	8.0

NC, not calculable.

ADF, which was reported by Yodoi et al,[14] is a novel lymphokine produced by HTLV-I-infected cell line cells, ATL-2. ADF is known to increase expression of Tac antigen on YT cells, which are derived from a patient with lymphoblastic lymphoma with thymic involvement and express natural killer activity.[14-16] The mechanism of induction of Tac antigen by ADF is not clear; however, lack of IL 2 production by cells stimulated with ADF was recently demonstrated.[17] Thus it is possible that ADF acts in a direct way for the induction of Tac antigen. It is controversial whether ADF might be one of the IL 1-related lymphokines produced by T cells; however, our findings indicate that ADF is distinct from IL 1.

A number of lymphokines are known to be secreted from HTLV-I-infected cell line cells, including γ-IFN, macrophage-activating factor, colony-stimulating factor (CSF), interleukin 3 (IL 3), and differentiation-inducing factor.[12] Recently, DNA sequences of granulocyte-macrophage CSF (GM-CSF) were revealed using HTLV-II-infected cell line cells, Mo.[25] GM-CSF acts on myeloid progenitor cells and stimulates proliferation and maturation of these cells to mature neutrophils.[26] IL 3 has CSF activities.[27] However, the actions of these factors on fresh leukemic cells have not been well defined. Furthermore, ADF does not have IL 3 activities or CSF activities when cells from mice are used as target cells (unpublished observations, November 1985). Further experiments to characterize and purify ADF are now in progress, and we hope that they will answer these questions.

This report demonstrates that factor(s) secreted from HTLV-I-infected cells enhanced Tac antigen expression and promoted proliferation of fresh myeloid leukemic cells. MT-1 cells, infected with HTLV-I, caused neutrophilia and extramedullar granulopoiesis when transplanted to hamsters.[13] We have also frequently observed absolute neutrophilia in patients with ATL (unpublished observations, June

1985). ADF may, therefore, be involved in the development of neutrophilia in ATL. If this is the case, ADF would be also helpful for establishing cell lines of myeloid leukemic cells and would aid in developing insight to the biology of these leukemic cells. Attempts to establish cell lines from FI cells using ADF are under way.

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REFERENCES

1. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. French-American-British (FAB) co-operative group. Br J Haematol 33:451, 1976

2. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

3. Guyre PM, Morganelli PM, Miller R: Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. J Clin Invest 72:393, 1983

4. Perussia B, Dayton ET, Lazarus R, Fanning V, Trinchieri G: Immune interferon induces the receptors for monomeric IgG1 on human monocytic and myeloid cells. J Exp Med 158:1092, 1983

5. Ball ED, Guyre PM, Shen L, Glynn JM, Maliszewski CR, Baker PE, Fanger MW: Gamma interferon induces monocytoid differentiation in the HL 60 cell line. J Clin Invest 73:1072, 1984

6. Hattori T, Pack M, Bougnoux P, Chang ZL, Hoffman T: Interferon-induced differentiation of U937 cells: Comparison with other agents that promote differentiation of myeloid or monocyte-like cell lines. J Clin Invest 72:237, 1983

7. Perussia B, Dayton ET, Fanning V, Thiagarajan P, Hoxie J, Trinchieri G: Immune interferon and leukocyte conditioned medium induce normal and leukemic myeloid cells to differentiate along the monocytic pathway. J Exp Med 158:2058, 1983

8. Koeffler HP, Bar-Eli M, Territo M: Phorbol diester-induced macrophage differentiation of leukemic blasts from patients with human myelogenous leukemia. J Clin Invest 66:1101, 1980

9. Yodoi J, Takatsuki K, Masuda T: Two cases of T-cell chronic leukemia in Japan. N Engl Med 290:572, 1974

10. Takatsuki K, Uchiyama T, Sagawa K, Yodoi J: Adult T cell leukemia in Japan, in Seno S, Takaku F, Irino S (eds): Topics in Hematology. Amsterdam, Excerpta Medica, 1977, p 73

11. Hattori T, Uchiyama T, Toibana T, Takatsuki K, Uchino H: Surface phenotype of Japanese ATL cells characterized by monoclonal antibodies. Blood 58:645, 1981

12. Salahuddin SZ, Markham PD, Lindner SG, Gootenberg J, Popovic M, Hemmi H, Sarin PS, Gallo RC: Lymphokine production by cultured human T cells transformed by human T-cell leukemia-lymphoma virus-I. Science 223:703, 1984

13. Miyoshi I, Kubonishi I, Sumida M, Yoshimoto S, Hiraki S, Tsubota T, Kobashi H, Lai M, Tanaka T, Kimura I, Miyamoto K, Sato J: Characteristics of a leukemic T-cell line derived from adult T-cell leukemia. Jpn J Clin Oncol 9:485, 1979 (suppl)

14. Yodoi J, Teshigawara K, Uchiyama T, Wano Y, Tsudo M, Nikaido T, Smith KA, Maeda M: Adult T-cell leukemia/lymphoma-derived factor augmenting TCGF (IL-2) receptor expression, in Gallo RC, Essex ME, Gross L (eds): Human T-cell Leukemia-Lymphoma Viruses. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, 1984, p 255

15. Teshigawara K, Maeda M, Nishino K, Nikaido T, Uchiyama T, Tsudo M, Wano Y, Yodoi J: Adult T cell leukemia cells produce a lymphokine that augments interleukein 2 receptor expression. J Mol Cell Immunol 2:17, 1985

16. Yodoi J, Teshigawara K, Nikaido T, Fukui K, Noma T, Honjo T, Takigawa M, Sasaki M, Minato M, Tsudo M, Uchiyama T, Maeda M: TCGF (IL-2)-receptor inducing factor(s). I. Regulation of IL 2 receptor on a natural killer-like cell line (YT cells). J Immunol 134:1623, 1985

17. Okada M, Maeda M, Tagaya Y, Taniguchi Y, Teshigawara K, Yoshiki T, Diamantstein T, Smith KA, Uchiyama T, Honjo T, Yodoi J: TCGF (IL-2)-receptor inducing factor(s). II. Possible role of ATL derived factor (ADF) on constitutive IL-2 receptor expression of HTLV-I(+) T cell lines. J Immunol 135:3995, 1985

18. Uchiyama T, Broder S, Waldmann TA: A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac$^+$ cells. J Immunol 300:1393, 1981

19. Leonard WJ, Depper JM, Uchiyama T, Smith KA, Waldmann TA, Greene WC: A monoclonal antibody that appears to recognize the receptor for T cell growth factor: Partial characterization of the receptor. Nature 300:267, 1982

20. Maeda M, Shimizu A, Ikuta K, Okamoto H, Kashihara M, Uchiyama T, Honjo T, Yodoi J: Origin of human T-lymphotropic virus I-positive T cell lines in adult T cell leukemia. Analysis of T cell receptor rearrangement. J Exp Med 162:2169, 1985

21. Goldstein G, Irene SS, Chase MW: Studies on fluorescent antibody staining. I. Non-specific fluorescence with fluorescein-coupled sheep anti-rabbit globulins. J Exp Med 114:89, 1961

22. Wano Y, Uchiyama T, Fukui K, Maeda M, Uchino H, Yodoi J: Characterization of human interleukin 2 receptor (Tac antigen) in normal and leukemic T cells. J Immunol 132:3005, 1984

23. Herrmann F, Cannistra SA, Levine H, Griffin JD: Expression of interleukin 2 receptors and binding of interleukin 2 by gamma interferon-induced human leukemic and normal monocytic cells. J Exp Med 162:1111, 1985

24. Uchiyama T, Hori T, Tsudo M, Wano Y, Umadome H, Tamori S, Yodoi J, Maeda M, Sawami H, Uchino H: Interleukin-2 receptor (Tac antigen) expressed on adult T cell leukemia cells. J Clin Invest 76:446, 1985

25. Wong GW, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, Clark SC: Human GM-CSF: Molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810, 1985

26. Burgess AW, Camakaris J, Metcalf D: Purification and properties of colony-stimulating factor from mouse lung-conditioned medium. J Biol Chem 252:1998, 1977

27. Ihle JN, Keller J, Oroszlan S, Henderson LE, Copeland TD, Fitch F, Prystowsky MB, Goldwasser E, Schrader JW, Palaszynski E, Dy M, Lebel B: Biological properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P cell stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. J Immunol 131:282, 1983

Refined Chromosome Analysis as an Independent Prognostic Indicator in De Novo Myelodysplastic Syndromes

By Jorge J. Yunis, Robert E. Rydell, Martin M. Oken, Mark A. Arnesen, Mary G. Mayer, and Michael Lobell

In a study of 56 consecutive adult patients with de novo myelodysplastic syndromes (MDS), all cases were successfully analyzed with two refined chromosome banding techniques. Most patients (44 of 56, 79%) were found to have a chromosome defect. The majority of these patients had a recurrent loss of chromosomal material rather than a reciprocal translocation or inversion, as commonly found in acute leukemia. The three largest chromosomal categories found were associated with a wide range of survival. Twelve patients (21%) had normal chromosomes, a stable clinical course, and long survival (median follow-up time of 49 months, with all patients alive). Nine patients had in common a single chromosome defect resulting in either monosomy 7 or deletion 7q. They had a median survival of 12 months, and four died of acute nonlymphocytic leukemia (ANLL). Of 12 patients with complex defects, 11 had a complete or partial loss of a chromosome 5 and a complete or partial loss of the long arm of a chromosome 7 or 20. They had a poor median survival of four months, and six patients died of ANLL. Although the French-American-British (FAB) classification was also found to have some prognostic value, FAB subgroups were chromosomally heterogeneous and showed less dramatic differences in median survival than the larger chromosomal subgroups. We have shown, for the first time, that a refined chromosomal analysis is an independent prognostic indicator in de novo MDS and may be helpful in establishing therapeutic approaches in this difficult group of heterogeneous disorders.

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DE NOVO MYELODYSPLASTIC SYNDROMES (MDS) represent a prognostically diverse group of dyspoietic disorders[1] that appear in patients without a prior history of chemotherapy or radiotherapy.[2] They are most commonly seen in patients over age 60,[1] with a male-female ratio of approximately 1.6:1.0,[1] and are characterized by blood cytopenia and dysplastic changes of hematopoietic cells.[1,3,4] In most patients, the marrow is hypercellular and one or more cell lines have signs of dyspoiesis.[3] Approximately half the patients either develop acute nonlymphocytic leukemia (ANLL) or die of marrow failure within two years of diagnosis. The other patients have a milder clinical course and longer survival.[1,5]

De novo MDS has long been considered an untreatable disease; however, new therapeutic possibilities for patients have been developed during the past few years.[1,6-9] There is still reluctance to treat such patients with mild or aggressive chemotherapeutic regimens, since only about 20% progress to frank leukemia.[10-12] Also, it is often not clear which patients may be good candidates for treatments that may induce cell differentiation,[6-9] or which patients may have a prolonged and relatively benign course best managed with supportive therapy.[1]

A useful classification of MDS has been developed by the French-American-British (FAB) Cooperative Group.[3] Following the FAB criteria, five subgroups are found: refractory anemia (RA), refractory anemia with excess ring sideroblasts (RARS), refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEB-T), and chronic myelomonocytic leukemia (CMML).

Despite significant hematologic,[3] cytogenetic,[4,13,14] and therapeutic[6-9] advances, no generally accepted routine approach exists for determining prognosis and therapeutic options in de novo MDS.[1] In this study, we report a consecutive five-year study of de novo MDS in which refined chromosomal analysis and careful clinical and cytologic examination have been used. Our results allow us to define chromosomal analysis as an independent prognostic indicator in this difficult group of disorders.

MATERIALS AND METHODS

Between July 1980 and August 1985, 56 adult patients with de novo MDS were seen at the hematology/oncology sections of the University of Minnesota Hospitals and affiliated Abbott-Northwestern and United Hospitals and the Veterans Administration and Hennepin County Medical Centers. The series represented consecutive cases studied in the laboratory but not necessarily consecutive admissions.

For each patient, MDS was classified on the basis of the morphology of Wright's stained peripheral blood smears, bone marrow aspirates, particle sections, and bone marrow core biopsies. The FAB Acute Leukemia Cooperative Study Group criteria were used.[3] When necessary, standard cytochemical studies were performed with peripheral blood or bone marrow aspirates or both to help determine the origin of abnormal cells.[15] Among patients found to have blood cytopenia involving one or more of the formed elements of the blood, those with a marrow blast count of less than 30% and significant blood or marrow dyspoiesis of one or several cell types were classified as MDS. Patients with MDS had a hypercellular or normocellular bone marrow in 52 of 56 cases and blood cytopenia in one or more cell lines, involving neutrophils of $<1{,}500$ cells per microliter, platelets of $<150{,}000/\mu L$, and hemoglobin of <12 g/dL. Patients with a prior history of chemotherapy or radiotherapy (secondary or therapy-related MDS) were not included.

Following informed consent, high-resolution chromosome banding patterns at the 400- to 850-band stages were determined from 1 to 3 mL of bone marrow obtained from each patient. Two chromosome techniques were used simultaneously: a cell synchronization technique with 17-hour exposure of cells to 10^{-7} mol/L methotrex-

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ate[16] and an unsynchronized technique with exposure of cells to 10^{-5} mol/L thymidine for six hours at the end of culture.[17] Twenty to 50 mitoses were analyzed, whenever possible. Chromosomal abnormalities were described according to the short version of the 1971 Paris Conference,[18] using the high-resolution chromosome nomenclature.[19] An abnormal clone was defined by the presence of an identical defect in two or more mitoses.

The Kaplan-Meier procedure was used to determine median survival or median follow-up time; the Breslow test, to assess early differences in survival; and the Mantel-Cox test, for late differences.[20]

RESULTS

We analyzed high-resolution chromosomes of bone marrow specimens from 56 consecutive adult patients with de novo MDS who had been classified using FAB morphological criteria. There were 15 women and 41 men (14 from the Veterans Administration Medical Center) ranging in age from 17 to 98 years, with a mean age at initial diagnosis of 62 years (Table 1). All patients were studied at initial diagnosis except for patients 5, 10, 11, 12, 14, 20, 25, 35, 36, 39, 47, 50, 54, and 55 (Table 2). Overall, the 56 patients had a median follow-up of 24 months (29 still alive); 15 of them (26%) progressed to ANLL and 25 (45%) died within two years of diagnosis. All patients were treated symptomatically until RAEB-T or ANLL developed. Four patients at the RAEB-T stage (patients 25, 53, 54, and 56) were treated with high-dose cytosine arabinoside (hi ara-C, 3 g/m^2 intravenously every 12 hours for four days),[8] and all showed a complete disappearance of bone marrow blasts of at least six to eight months duration but with persistence of MDS features. Four other patients at the ANLL stage (patients 38, 47, 52, and 55) were treated with low-dose ara C (low ara-C, 10 mg/m^2 subcutaneously every 12 hours for 21 days).[7] Two (patients 38 and 52) had a partial remission of ten months' and one month's duration, respectively. The other two showed no response (Table 2). Patients were also subdivided according to the FAB classification as follows: RARS, 13 patients; RA, 13; RAEB, 23; RAEB-T, 4; and CMML, 3. Differences were observed among FAB subgroups in their tendency to transform to ANLL (three cases of RA [23%], no cases of RARS, nine of RAEB [39%], one of four RAEB-T, and two of three CMML). Among the three largest FAB subgroups, RARS showed a median follow-up time of 24 months (11 of 13 patients still alive), and RA and RAEB showed a median survival of 29 and 14 months, respectively.

A history of carcinogenic or mutagenic exposure was found in 21% of the 56 patients (Table 2; patients 5, 12, 13, 20, 25, 28, 31, 33, 36, 52, 55, and 56). For example, patients 5, 12, 13, 20, 25, 31, 33, 36, 52, and 56 were occupationally involved with solvents (eg, patient 36 was chronically exposed to benzene) and patient 55 had accidental exposure to gamma radiation (atomic bomb observer).

Specimens from all 56 patients were successfully analyzed in the initial chromosome study, and 79% showed a chromosomal abnormality. The specific defects and clinical course are outlined in Tables 1 and 2, respectively. Thirty-nine of the 56 patients (70%) showed one of seven types of recurrent defects: deletion 5q, monosomy 7 or deletion 7q, deletion 7p, trisomy 8, deletion 9q, deletion 20q, or complex (three or more) defects often involving a deletion 5q, 7q, and 20q (Table 1, Fig 1). Miscellaneous nonrecurring chromosomal defects were found in five patients (patients 40 through 44).

The three largest chromosomal groups found were represented by patients with normal chromosomes, monosomy 7 or partial deletion 7q, and complex defects (Table 1). Twelve patients with normal chromosomes, classified under FAB as RARS (nine), RA (two), or RAEB (one), are all alive with a median follow-up of 49 months and receive only symptomatic treatment (Table 2). A monosomy 7 or partial deletion 7q as a single chromosomal defect was observed in nine

Table 1. Initial Chromosomal Findings of 56 Patients With De Novo MDS

Case No.	Sex/Age	FAB	Mitoses Analyzed		Chromosomal Findings
			Abnormal	Normal	
Normal chromosomes					
1	M/65	RA	0	50	46,XY
2	M/63	RARS	0	50	46,XY
3	F/57	RARS	0	50	46,XX
4	F/65	RARS	0	42	46,XX
5	F/61	RARS	0	50	46,XX
6	M/80	RA	0	55	46,XY
7	M/72	RAEB	0	55	46,XY
8	M/75	RARS	0	50	46,XY
9	F/24	RARS	0	50	46,XX
10	M/75	RARS	0	50	46,XY
11	M/62	RARS	0	51	46,XY
12	M/64	RARS	0	45	46,XY
Del 5q					
13	F/48	RA	5	7	46,XX,del(5)(q31q35)
14	M/62	RA	8	32	46,XY,del(5)(q31q35)
15	M/32	RA	16	4	46,XY,del(5)(q31q35)
16	F/54	RA	5	45	46,XX,del(5)(q31q35)
17	F/57	RA	37	7	46,XX,t(2;3)(p23;q27),del(5)(q22q35)
Monosomy 7 or del 7q					
18	M/58	RA	2	105	46,XY,del(7)(q31.2q36)

Table 1. Initial Chromosomal Findings (Cont'd)

Case No.	Sex/Age	FAB	Mitoses Analyzed		Chromosomal Findings
			Abnormal	Normal	
19	M/76	RA	2	18	46,XY,del(7)(q31.2q36)
20	M/43	RAEB-T	9	6	45,XY,−7
21	M/76	RA	3	57	45,XY,−7
22	F/66	RAEB	20	0	46,XX,del(7)(q34q36)
23	M/76	RAEB-T	2	18	46,XY,del(7)(q31.2q36)
24	F/65	CMML	8	5	45,XX,−7
25	M/36	RARS	17	20	46,XY,+der(1)t(1;7)(p11.2;p11.2),−7
26	F/17	RAEB	8	5	46,XX,+der(1)t(1;7)(p11.2;p11.2),−7
Del(7)(p11.2p22)					
27	M/71	RAEB	26	4	46,XY,del(7)(p11.2p22)
28	M/40	RAEB	14	1	46,XY,−7,+der(7)t(7;9)(p11.2;p11.2)
Del(9)(q13q22)					
29	M/79	RAEB-T	7	30	45,XY,del(9)(q13q22),−17
30	F/83	RAEB	8	10	46,XX,del(5)(q13q35),del(9)(q13q22)
Del 20(q12q13)					
31	M/36	RAEB	5	7	46,XY,del(20)(q12q13)
32	M/58	RAEB	10	7	46,XY,del(20)(q12q13)
33	M/60	RARS	20	3	46,XY,del(20)(q12q13)
			2		47,XY,+8,del(20)(q12q13)
34	F/78	RAEB	13	0	46,XX,del(20)(q12q13)
Trisomy 8					
35	M/65	RAEB	9	0	47,XY,+8
36	M/64	RAEB	2	108	47,XY,+8
37	F/63	RAEB	8	5	47,XX,+8
38	M/68	RAEB	6	2	47,XY,+8
39	M/76	RA	4	10	47,XY,+8
Miscellaneous defects					
40	M/59	RAEB	6	9	45,XY,t(16;17)(p11.2;p11.2)del(16)(p11.2q24) del(17)(p11.2p23)
41	M/63	RAEB	2	38	47,XY,del(1)(q21q44)
42	M/70	RA	2	8	46,xy,del(11)(q14.2q23)
43	M/59	RAEB	3	42	46,XY,dup inv(1)(q21q42),del(10)(p13p15)
44	M/98	CMML	18	0	45,X0
			2		45,X0,dup(12)(q13q24.1)
Complex chromosomal defects					
45	M/74	RAEB	9	4	45,X0,del(5)(q13q35),del(7)(q35q36),del(20)(q12q13), t(1;11)(q42;q13),del(2)(q33q35),t(4;15)(q35;q15), −15,del(16)(p31),del(17)(q21q25),del(18)(q12.2q23),dm
46	M/64	RARS	17	1	45,X0,del(5)(q31q35),del(7)(q22q36),del(20)(q13), del(14)(q11.2q21)*
47	F/77	RAEB-T	9	1	44,XX,del(5)(q13q35),t(20;21;22)(q11.2;q11.2;p11.1), t(2;6)(p23;p21.3),−17,−18,,sm r
48	M/76	RA	7	3	46,XY,del(5)(q13q31),−7,t(1;2)(q21;p21), t(1;11)(p22;p15.1),+8,t(12;?)(p13;?),t(15;?)(p11.2;?)
49	M/78	CMML	10	1	46,XY,t(1;7)(q21;q22)del(7)(q22q36), t(8;12)(q21;q24),del(10)(p13p15),del(21)(q21q22)
50	M/50	RAEB	11	2	49,XY,del(5)(q22q31),−7,+20, del(1)(p32p36),del(1)(q25q44),+18,+19
51	M/52	RAEB	10	0	44,XY,del(5)(q31q35),−7,−20
52	M/35	RAEB	6	4	46,XY,del(5)(q15q31),del(7)(q22q35), del(1)(p12p22),del(4)(q21q31),del(15)(q24q26)*
53	M/57	RAEB	11	0	44,XY,−5,−20,del(3)(q25q27),−11,+12,+18,−21*
54	F/65	RAEB	19	0	47,XX,del(5)(q15q35),del(7)(q31q36), del(4)(q21q27),t(11;14)(p11.2;q13),t(12;15)(q12;p11.2),r*
55	M/60	RAEB	9	1	44,X0,del(5)(q31q35),−7,del(20)(q12q13), t(12;22)(p11.2;q11.2),t(21;?)(q22;?)
56	M/64	RARS	11	9	43,XY,del(5)(q13q35),−20,t(6;16)(p25;q21),−18

Description of recurrent defects involving chromosomes 5, 7, and 20 have been arranged following chromosome number and sex complement, to simplify finding these defects.

*Some variability of the abnormal chromosome complement was observed in several mitoses.

Table 2. Clinical Data of 56 Patients With De Novo MDS

Case No.	Original Diagnosis		Chromosome Study	Disease Evolution FAB	Survival (mo)	Exposure to Carcinogens, History of Infections, Chemotherapy for MDS
	FAB	Date				
Normal chromosomes						
1	RA	7/85	7/85		1*	
2	RARS	1/85	1/85		7*	
3	RARS	5/84	5/84 1/85		16*	
4	RARS	8/83	8/83		24*	
5	RARS	8/83	5/84		24*	Solvents (furniture stripping, past 10 yr)
6	RA	7/81	7/81 6/83		48*	
7	RAEB	7/81	7/81		49*	
8	RARS	1/81	1/81 8/85		55*	
9	RARS	7/80	7/80		60*	
10	RARS	2/80	8/81 1/82 3/83		66*	
11	RARS	1/78	5/84		79*	
12	RARS	10/78	4/84 11/84		82*	Solvents (airline maintenance, past 30 yr)
Deletion 5q						
13	RA	3/85	3/85		5*	Solvents (painter, past 16 yr)
14	RA	6/82	9/84 1/85		27*	
15	RA	11/80	11/80		48*	
16	RA	7/80	7/80 5/83		60*	
17	RA	5/84	5/84			Urinary tract infection
			8/84	ANLL-M1	9	
Monosomy 7 or del 7q						
18	RA	3/82	3/82		3	
19	RA	3/83	3/83			
			6/83	ANLL-M1	8	
20	RAEB-T	11/84	8/85		9	Herbicides, pesticides (past 11 yr)
21	RA	6/82	6/82 3/83		10	Multiple infections
22	RAEB	11/83	11/83			
			6/84	ANLL-M2	12	
23	RAEB-T	12/83	12/83		13	Multiple infections
24	CMML	2/84	2/84			
			11/84	ANLL-M4	14	
25	RARS	5/84	6/84			Herbicides, pesticides, solvents (past 5 yr exposure); hi ara-C (CR 8 mo+)
			7/84	RAEB		
			1/85	RAEB-T	15*	
26	RAEB	8/81	8/81			
			10/82	ANLL-M5	16	
Deletion 7p						
27	RAEB	6/83	6/83		8*	
28	RAEB	4/82	4/82			Dioxin (Agent Orange, 1966); multiple infections)
			2/83	ANLL-M2	10	
Deletion 9q						
29	RAEB-T	6/84	6/84 8/84		2	
30	RAEB	1/82	1/82			
			4/82	ANLL-M2	8	
Deletion 20q						
31	RAEB	3/85	3/85		5*	Solvents (mechanic, past 18 yr)
32	RAEB	2/85	2/85		6*	
33	RARS	10/84	10/84			Solvents (furnace repairman, past 30 yr)
			1/85	RAEB		
			8/85	RAEB-T	9	

Table 2. Clinical Data (Cont'd)

Case No.	Original Diagnosis FAB	Original Diagnosis Date	Chromosome Study	Disease Evolution FAB	Survival (mo)	Exposure to Carcinogens, History of Infections, Chemotherapy for MDS
34	RAEB	9/84	9/84			Multiple infections
			12/84		10.5*	
Trisomy 8						
35	RAEB	1/85	7/85		7*	
36	RAEB	9/80	12/81			Benzene, other solvents (past 10–15 yr exposure)
			1/82	RAEB-T	18	
37	RAEB	2/84	2/84			
			8/85	ANLL-M2	18*	
38	RAEB	8/83	8/83			Multiple infections; low ara-C (PR 10 mo)
			10/84	ANLL-M4	25	
39	RA	9/82	4/83		29	Recurrent infections
			4/84			
Miscellaneous defects						
40	RAEB	5/85	5/85		3*	
41	RAEB	4/85	4/85		4*	
42	RA	5/85	5/85		3*	
43	RAEB	9/84	9/84		11*	
44	CMML	2/83	2/83		16	
Complex chromosomal defects						
45	RAEB	11/84	11/84		1	
46	RARS	3/85	3/85		1.5	
			4/85	myelofibrosis		
47	RAEB-T	9/84	10/84			Recurrent infection; low ara-C (NR)
			12/84	ANLL-M2	2	
48	RA	9/81	9/81			
			12/81	ANLL-M1	3	
49	CMML	5/85	5/85			
			8/85	ANLL-M5	3	
50	RAEB	9/81	1/82			
			4/82	RAEB-T	3.5	
51	RAEB	2/85	2/85			
			5/85	RAEB-T	4	
52	RAEB	1/84	1/84			Solvents (furniture stripping)
			3/84			
			4/84			
			5/84	ANLL-M6	6	Low ara-C (PR 1 mo)
53	RAEB	11/84	11/84			
			1/85	RAEB-T	9*	Hi ara-C (CR 7 mo+)
54	RAEB	2/83	7/83			Multiple infections; hi ara-C (CR 7 mo)
			1/84	RAEB-T		
			4/84			
			8/84	ANLL-M4	13.5	
55	RAEB	11/82	1/83			Radiation (nuclear bomb, 1945–49); multiple infections; low ara-C (NR)
			1/84	ANLL-M4	14	
56	RARS	2/84	2/84			Solvents and fumes (salesman, 30-yr exposure); hi ara-C (CR 6 mo+)
			6/84	RAEB-T		
			12/84		19*	

Hi and low ara C, high- and low-dose cytosine arabinoside, respectively; CR, PR, and NR, complete, partial, and no remission of blastic transformation, respectively.

*Patient alive. Others died of hemorrhage, infection, or ANLL.

patients (18 through 26) classified as either RA (three), RARS (one), RAEB (two), RAEB-T (two), or CMML (one). These patients had a median survival of 12 months, four died of ANLL within eight to 16 months after diagnosis, and only one patient (25), who is on hi ara-C, is still alive (Table 2).

Twelve patients (45 through 56) were found to have three or more chromosomal defects (complex defects) and a median survival of four months. Six of these patients developed ANLL and the only two patients alive (53 and 56) are on hi ara-C. Eleven of the 12 patients carried a partial or complete loss of a chromosome 5; nine of 12 had a complete or partial deletion 7q; and six of 12, a complete or partial deletion 20q (Table 1). Patients with complex defects were

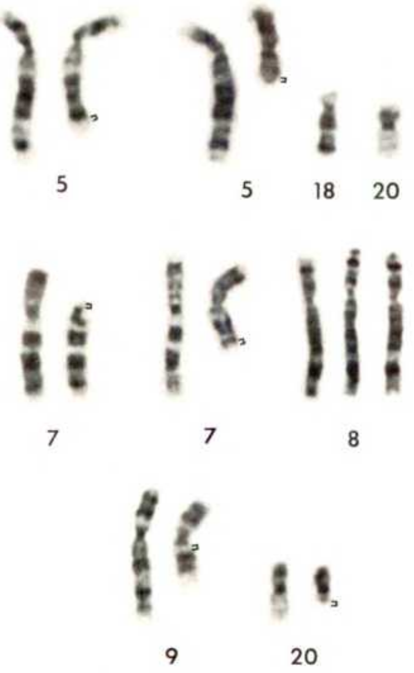

Fig 1. Selected G-banded chromosomes at the 600-band stage from patients with recurrent chromosomal defects, used to show representative abnormalities found in de novo MDS subgroups. Top row: RA with del (5) (q31q35), patient 14; RARS with complex chromosomal defects, including del (5) (q13q35), monosomy 18, and monosomy 20, patient 56. Middle row: RAEB with del (7) (p11.2p22), patient 27; RAEB-T with del (7) (q31.2q36), patient 23; RAEB with trisomy 8, patient 38. Bottom row: RAEB-T with del (9) (q13q22), patient 29; RAEB with del (20) (q12q13), patient 32.

variously diagnosed as RARS (two), RA (one), RAEB (seven), RAEB-T (one), or CMML (one) (Table 1).

Although RARS was found in four chromosomal categories, it is interesting that in our series, nine of 12 patients with normal chromosomes were RARS (Table 3). Also, although patients with RA were found in six chromosomal categories, all four patients with a single deletion 5q were RA (Table 3).

The 56 patients in this study were followed cytologically two to five times during the course of their disease. FAB diagnosis changed in 23 (41%), as noted in column 5 of Table 2. Thirteen of 18 patients (72%) with monosomy 7/deletion 7q either singly or with additional defects (patients 19, 22, 24 through 26, 46, 48 through 52, 54, and 55) experienced evolution to ANLL or to a clinically more severe MDS, while only ten of the other 38 patients (26%) experienced such evolution.

Among the 12 patients found to have normal chromosomes and a stable clinical and cytologic course (patients 1 through 12), five that were followed with chromosome studies (patients 3, 6, 8, 10, and 12) were found to retain their normal karyotype on repeat examination (Tables 2 and 4). This contrasts with 17 of 44 patients with a chromosomal defect who were followed with repeated analysis during an interval of 18 months, of whom 14 (82%) showed cytologic and chromosomal changes (Table 4). Further, four of these patients showed the additional presence of a deletion 5q, monosomy 7 or trisomy 8 superimposed on the previous chromosomal defect (patients 17, 21, 26, and 34), and in three others, the presence of a del 5q, −7 or +8 was found in all the cells examined, rather than an admixture of normal and abnormal cells found at diagnosis (patients 24, 28, and 38). Two patients with a single deletion 5q who were followed sequentially (patients 14 and 16) showed a stable clinical and cytologic course, contrasting with two patients who had a deletion 5q accompanied by either a t(2; 3) or a deletion 9q (Table 4; patients 17 and 30).

DISCUSSION

Using standard metaphase chromosome techniques (250- to 320-band stages), older as well as recent studies have consistently reported that only one third to one half of patients with de novo MDS have chromosomal defects (37% to 51%).[4,13,14,21] This, coupled with an absence of long-term

Table 3. Subgroups and Median Survival of De Novo MDS According to Chromosomal Findings and to FAB Classification

Chromosomal Findings	No. of Patients	FAB Classification					Median Survival (mo)
		RARS [13]	RA [13]	RAEB [23]	RAEB-T [4]	CMML [3]	
Normal chromosomes	12 (12)	9	2	1			49*
Single recurrent defects†							
del 5q	5 (4)		5				
−7/del 7q	9 (1)	1	3	2	2	1	12
del 7p	2 (1)			2			
del 9q	2 (0)			1	1		
del 20q	4 (3)	1		3			
+8	5 (2)		1	4			
Miscellaneous defects	5 (4)		1	3		1	
Complex defects	12 (2)	2	1	7	1	1	4
FAB median survival (mo)		24*	29	14			

Results shown represent median survival of major subgroups only. The number of patients still alive is shown in parentheses. The total number of patients in each FAB category is shown in brackets.

*Actually represents median follow-up time.

†Del 5q and del 20q categories have one patient each with an additional defect. Both patients with del 9q have two chromosomal defects.

Table 4. Chromosomal Evolution of 22 Patients With De Novo MDS

Case No.	FAB Classification		Chromosomal Defects		Interval (mo)
	Initial	Transformation	Initial	Reanalysis	
3	RARS	None	Normal	Normal	8
6	RA	None	Normal	Normal	23
8	RARS	None	Normal	Normal	55
10	RARS	None	Normal	Normal	17
12	RARS	None	Normal	Normal	7
14	RA	None	del 5q	del 5q	4
16	RA	None	del 5q	del 5q	9
17	RA	ANLL-M1	t(2;3), del 5q	t(2;3), del 5q, −7	3
19	RA	ANLL-M1	del 7q	del 7q (most cells)	3
21	RA	RAEB-T	−7	−7, +8	8
24	CMML	ANLL-M4	−7	−7 (all cells)	9
25	RARS	RAEB, RAEB-T	t(1;7), −7	t(1;7), −7 (most cells)	2, 8
26	RAEB	ANLL-M5	t(1;7), −7	t(1;7), −7, +8	14
28	RAEB	ANLL-M2	−7, der (7), t(7;9)	Same (all cells)	10
30	RAEB	ANLL-M2	del 5q, del 9q	del 5q, del 9q, t(1;4)	3
33	RARS	RAEB, RAEB-T	del 20q, +8	del 20q, +8 (most cells)	3, 10
34	RAEB	None	del 20q	del 20q, del 5q	3
37	RAEB	ANLL-M2	+8	+8, del 16q, del 17p	18
38	RAEB	ANLL-M4	+8	+8 (all cells)	14
52	RAEB	ANLL-M6	Complex	Complex, plus del 11p, dic 14, t(16;17)	4
54	RAEB	RAEB-T, ANLL-M4	Complex	Complex, plus −21, t(3;15)	6, 7
56	RARS	RAEB-T	Complex	Complex	4

follow-up clinical studies, has not made it previously possible to determine the routine prognostic value of chromosome studies in de novo MDS.[4,13,14,21]

It has been suggested that patients with all metaphases normal (NN) are less likely to develop ANLL and that no prognostic difference exists between patients who have all mitoses abnormal (AA) and those with an admixture of normal and abnormal mitoses (AN).[13] Such findings were confirmed in our study, since none of our NN patients developed ANLL (patients 1 through 12) and AA patients (22, 34, 35, 44, and 51 through 53) did not have a shorter survival than the median found in their corresponding chromosomal subgroups (Tables 1 and 2).

The ability to find a chromosomally abnormal clone in most de novo MDS was dramatically enhanced by our simultaneous use of two refined chromosome techniques (320- to 850-band stages) in each patient: a methotrexate cell-synchronization technique that was successfully performed in 53 of the 56 cases and an unsynchronized technique with thymidine exposure that was successful in 46 of the 56 cases. Using both techniques, all 56 patients were successfully analyzed, suggesting that all, or most, de novo MDS patients can be analyzed with this combined approach.

In the past, the value of several possible prognostic indicators in de novo MDS has been discussed.[1,5,10,11] In the absence of other useful routine parameters, the FAB diagnostic classification has been reported in some large retrospective studies to be the best prognostic guide available.[10,11]

The three main FAB subgroups in our series (RARS, RA, and RAEB) were also found to show differences in survival that have some prognostic importance, but such differences were not as marked and clinically useful as those observed in the three main chromosomal subgroups (normal chromosomes, monosomy 7 or deletion 7q, complex defects). For example, among the three main chromosome groups, it was found that there was a clear distinction in survival during early and late stages of the disease in the group with normal chromosomes when compared with those with either monosomy 7/del 7q or complex defects ($P = .0001$ and .00001, respectively) (Fig 2B). In the large FAB subgroups studied (RARS, RA, RAEB), early and late differences in survival were statistically significant only when RARS was compared with RAEB alone ($P = .05$ and .005, respectively). No significant difference was observed, however, when the three groups were compared with one another ($P = .1$ and .07, respectively) (Fig 2A).

Patients diagnosed as RARS with normal chromosomes had a highly favorable prognosis and stable clinical course (median follow-up time 55 months, with all nine patients still alive). In contrast, patients with RARS and monosomy 7, deletion 20q, or complex defects (25, 33, 46, and 56) may have a less promising prognosis, since all four patients with these characteristics evolved into a more aggressive course within one to four months and two died 1½ and nine months from diagnosis (Table 2). Such findings suggest that de novo MDS patients with RARS disease and normal chromosomes will probably have a long survival and should be treated symptomatically, while newly diagnosed RARS patients with one or more recurrent chromosomal defects may have a more aggressive disease and should be followed closely for possible evolution to a more aggressive course. At this point, intervention with chemotherapy may be indicated. Patients 25 and 56 were treated with hi ara-C and responded with a marrow blast remission when they were at the RAEB-T stage (Table 2).

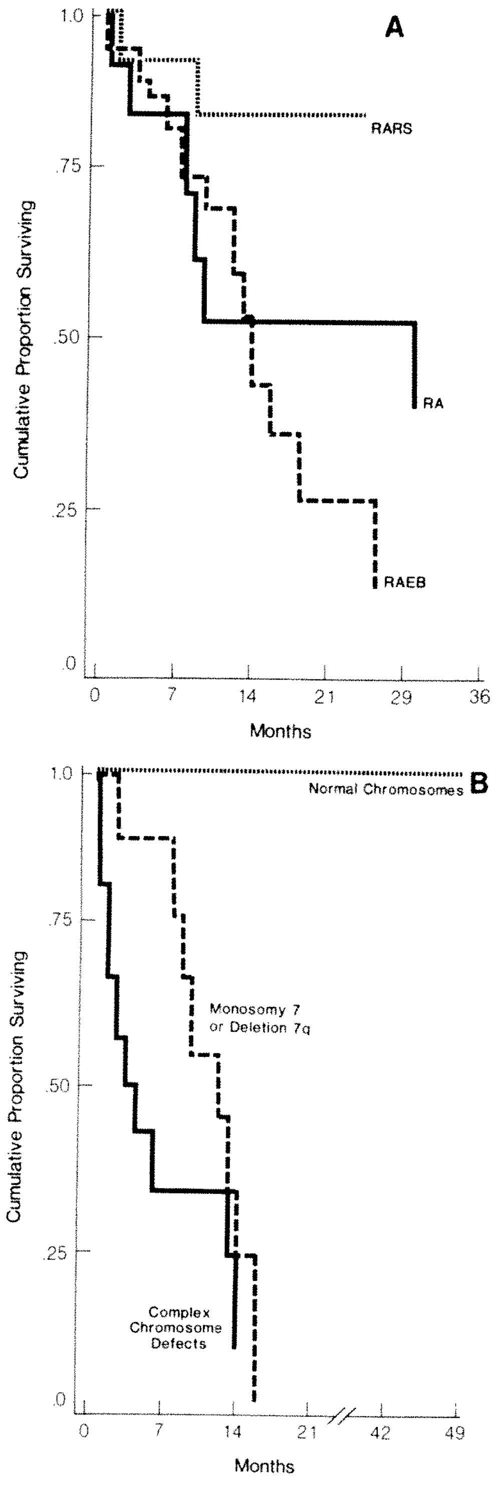

Fig 2. Survival curves of the three largest (A) FAB and (B) chromosome subgroups of de novo MDS.

Although RA has been traditionally considered to have good prognosis, recent reports indicate that 15% to 22% of RA patients may transform to ANLL.[10-12] In our study, we found that three of our 13 RA patients also developed ANLL (23%). Further, we observed that three of four RA patients found to have partial or complete monosomy 7 or complex defects developed ANLL or died within four months of diagnosis, the fourth dying at ten months (Table 2, patients 18, 19, 21, and 48). On the other hand, two RA patients with normal chromosomes and four with a single deletion 5q have remained clinically stable up to 60 months from diagnosis (Table 2). Because RA was a poorly defined entity in the original FAB classification report,[3] a large prospective study is indicated to separate, if possible, subgroups within this category.

As for RAEB, 13 of the 23 patients we studied evolved in one to 14 months to RAEB-T or ANLL, and 11 have died (Table 2; patients 22, 26, 28, 30, 36 through 38, 50 through 55; Fig 2). On the other hand, nine RAEB patients are clinically stable and have survived five to 49 months (Table 2; patients 7, 27, 31, 32, 34, 35, 40, 41, 43). These findings suggest that although approximately half the RAEB patients have a poor prognosis (<one year survival), the other half have a better prognosis (>one year survival; Fig 2A), as observed in a recent large series.[12] As with RA, we suggest that a combined FAB-chromosome study of a large number of patients may help to separate subgroups of RAEB that have different survivals.

In contrast to the more variable prognosis found in the three more common FAB subgroups (RARS, RA, RAEB; Fig 2A), the three main chromosomal subtypes found were noted to serve as a more uniform prognostic indicator in de novo MDS (Fig 2B). In addition to the finding of normal chromosomes as a good prognostic indicator already discussed, complex defects (three or more defects, including del 5q, del 7q or monosomy 7 and del 20q or monosomy 20) imparted a strikingly poor prognosis (Table 2, Fig 2B) regardless of FAB classification (RARS, RA, RAEB, RAEB-T, CMML). Except for one RAEB patient who died within one month of diagnosis, the other 11 patients diagnosed as having complex chromosomal defects quickly transformed to a more aggressive disease and six died of leukemia (Table 2). The median survival for this group was only four months, and the two patients still alive (53 and 56) are on hi ara-C.

Monosomy 7 or a partial loss of the long arm of a chromosome 7 as a single defect, found in nine cases, also imparted poor prognosis and a high probability of evolution to ANLL, regardless of the FAB classification (RARS, RA, RAEB, RAEB-T, CMML) (Table 2, Fig 2B). Such variablility of monosomy 7 in MDS subtypes, as well as evolution to one of various ANLL subgroups (M1, M2, M4 through M6), has been noted.[22,23] The nine patients showed a single chromosomal defect with a common deletion of the segment 7q34q36 and a median survival of 12 months (the only living patient is being treated with hi ara-C). Among them, seven had a simple monosomy 7 or deletion 7q and two had a t(1;7)(p11.2;p11.2) replacing one chromosome 7. As a result, the patients were trisomic for 1q and monosomic for 7q, as recently reported in 12 patients in the Netherlands.[24] Children and adolescents with monosomy 7, as in adults, also rapidly convert to ANLL, but this defect is often found in association with a myeloproliferative syndrome and not MDS.[25]

Besides the three larger chromosomal subgroups defined (normal chromosomes, del 7q, and complex defects with two or more recurrent deletions often involving del 5q and 7q), six smaller chromosomal subtypes were found. They included a single deletion 5q, a deletion 7p, trisomy 8, deletion 9q, deletion 20q, and miscellaneous defects. Interestingly, in de novo MDS, we found that of 44 patients with abnormal chromosomes, all but six (86%) had a loss of chromosomal material. This strongly suggests that a single or multiple deletion mechanism is most common in de novo MDS.

In fact, cases with a single or combined deletion 5 or 7 represent half of all cases studied, and their median survival was only eight and 8½ months, respectively. This suggests that such defects are generally associated with poor prognosis, except for patients with a single del 5q (patients 13 through 16), who had a much longer survival, as recently reported.[26,27]

Our chromosomal analysis of de novo MDS allows these disorders to be viewed more accurately in relation to de novo ANLL. Simple trisomy 8 or loss (complete or partial) of a chromosome 5 or 7 is found in less than one fifth of de novo ANLL, whereas specific translocations or an inversion 16 are found in more than one third.[28] This suggests that diseases with the latter chromosomal defects have at most a transient preleukemic period, which is seldom detected clinically. Disorders characterized by trisomy 8 or the complete or partial loss of a chromosome 5 or 7, however, may present either as frankly leukemic[28] or as preleukemic with MDS.[13,14] Similar chromosomal defects are commonly found in secondary MDS and ANLL, but more often in association with additional chromosomal defects.[29-31] In fact, evolution of de novo MDS to a more aggressive clinical course, including ANLL, was often found to be associated with the acquisition of additional chromosome defects (Table 4).

In our study of de novo MDS, it was observed that 21% of patients have had a history of chronic exposure to potential carcinogens in their occupation or the environment. Because patients with secondary MDS by definition represent cases exposed to chemotherapy or radiotherapy, these two groups of myelodysplasia may possibly represent a spectrum of related disorders caused by carcinogenic exposure. Nevertheless, it should be made clear that a clinical history of possible occupational exposure to potential carcinogens does not constitute evidence of cause-effect relationship, contrary to the known high-level exposure of patients who have been treated with chemotherapy or radiotherapy for a previous disease (secondary or therapy-related MDS).

Furthermore, despite the observation that some de novo MDS patients with possible carcinogenic exposure and those with secondary MDS may have similar clinical and chromosomal features, our study suggests that approximately one fifth of all de novo MDS patients have a stable clinical course and normal chromosomes. This contrasts to secondary MDS, in which only 4% to 11% of the cases are reported to have normal chromosomes when standard techniques are used.[29-31] Also, patients with secondary MDS more often have complex chromosomal defects, evolve into leukemia, and have a short median survival of less than four months.[29-31]

For the more aggressive types of MDS, new treatment modalities, such as the use of hi or low ara-C, are being investigated.[6-8] In this respect, four of our patients in the RAEB-T phase of the disease were treated with hi ara-C and had a blast-free remission lasting for at least six to eight months, with three patients still free of blasts in the marrow. This moderately increased the survival time of patients in the poor prognosis chromosome categories (Table 2). As noted by other investigators,[8] however, frank dyspoiesis has persisted and toxicity was not infrequent. In our limited experience, low ara-C was less effective, with two patients treated during the final ANLL stage of the disease having no response (patients 47 and 55) and two having a partial response with persistence of dyspoiesis and a slight elevation of marrow blasts (patients 38 and 52). This again correlates with results of recent larger series.[6] Additional studies are needed to assess the value of hi and low ara-C using specific chromosomal subgroups.

As in ANLL, in which chromosomal analysis is used in addition to morphological studies to identify reliable prognostic subtypes,[28] we have found that a refined and detailed analysis strongly enhances the identification of prognostic subgroups in de novo MDS (Table 2, Fig 2) and should be used routinely at diagnosis to design therapeutic modalities in a more precise manner.

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REFERENCES

1. Hoelzer D, Ganser A, Heimpel H: "Atypical" leukemias: Preleukemia, smoldering leukemia and hypoplastic leukemia: Recent results. Cancer Res 93:69, 1984

2. Fourth International Workshop on Chromosomes in Leukemia: A prospective study of acute nonlymphocytic leukemia. Cancer Genet Cytogenet 11:249, 1984

3. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

4. Nowell PC: Cytogenetics of preleukemia. Cancer Genet Cytogenet 5:265, 1982

5. Weisdorf DJ, Oken MM, Johnson GJ, Rydell RE: Chronic myelodysplastic syndrome: Short survival with or without evolution to acute leukaemia. Br J Haematol 55:691, 1983

6. Griffin JD, Spriggs D, Wisch JS, Kufe DW: Treatment of preleukemic syndromes with continuous intravenous infusion of low-dose cytosine arabinoside. J Clin Oncol 3:982, 1985

7. Winter JN, Variakojis D, Gaynor ER, Larson RA, Miller KB: Low-dose cytosine arabinoside (ara-C) therapy in the myelodysplastic syndromes and acute leukemia. Cancer 56:443, 1985

8. Herzig RA, Lazarus HM, Wolff SN, Phillips GL, Herzin GP: High-dose cytosine arabinoside therapy with and without anthracycline antibiotics for remission reinduction of acute nonlymphoblastic leukemia. J Clin Oncol 3:992, 1985

9. Gold EJ, Mertelsmann RA, Itri LM, Gee T, Arlin Z, Kempin S, Clarkson B, Moore MAS: Phase I clinical trial of 13-cis-retinoic acid in myelodysplastic syndromes. Cancer Treat Rep 67:981, 1983

10. Coiffier B, Adeleine P, Viala JJ, Byron PA, Fiere D, Gentilhomme O, Vuvan H: Dysmyelopoietic syndromes: A search for prognostic factors in 193 patients. Cancer 52:83, 1983

11. Foucar K, Langdon RM, Armitage JO, Olson DB, Carroll TJ Jr: Myelodysplastic syndromes: A clinical and pathologic analysis of 109 cases. Cancer 56:553, 1985

12. Juneja SK, Imbert M, Jouault H, Scoazec J-Y, Sigaux F, Sultan C: Haematological features of primary myelodysplastic syndromes (PMDS) at initial presentation: A study of 118 cases. J Clin Pathol 36:1129, 1983

13. Second International Workshop on Chromosomes in Leukemia. Chromosomes in preleukemia. Cancer Genet Cytogenet 2:108, 1980

14. Knapp RH, Dewald GW, Pierre RV: Cytogenetic studies in 174 consecutive patients with preleukemic or myelodysplastic syndromes. Mayo Clin Proc 60:507, 1985

15. Williams WJ, Beutler E, Erslev AJ, Lichtman MA (eds): Hematology (ed 3). New York, McGraw-Hill, 1983, p 1646

16. Yunis JJ: New chromosome techniques in the study of human neoplasia. Hum Pathol 12:540, 1981

17. Yunis JJ, Lewandowski R: High-resolution cytogenetics, in Finley SC, Finley WH, Flowers CE Jr (eds): Birth Defects: Clinical and Ethical Considerations. Original article series, vol 19. New York, Alan R. Liss, 1983, p 11

18. ISCN: An international system for human cytogenetic nomenclature. Cytogenet Cell Genet 21:309, 1978

19. ISCN: An international system for human cytogenetic nomenclature—high-resolution banding. Cytogenet Cell Genet 31:1, 1981

20. Elandt-Johnson RC, Johnson NL: Survival Models and Data Analysis. New York, John Wiley, 1980

21. Tricot G, Vlietinck R, Boogaerts MA, Hendrickx B, De Wolf-Peeters C, Van Den Berghe H, Verwilghen RL: Prognostic factors in the myelodysplastic syndromes: Importance of initial data on peripheral blood counts, bone marrow cytology, trephine biopsy and chromosome analysis. Br J Haematol 60:19, 1985

22. Borgström GH, Teerenhovi L, Vuopio P, de la Chapelle A, Van Den Berghe H, Brandt L, Golomb HM, Louwagie A, Mitelman F, Rowley JD, Sandberg AA: Clinical implications of monosomy 7 in acute nonlymphocytic leukemia. Cancer Genet Cytogenet 2:115, 1980

23. Pasquali F, Bernasconi P, Casalone R, Fraccaro M, Bernasconi C, Lazzarino M, Morra E, Alessandrino EP, Marchi MA, Sanger R: Pathogenetic significance of "pure" monosomy 7 in myeloproliferative disorders. Analysis of 14 cases. Hum Genet 62:40, 1982

24. Scheres JMJC, Hustinx TWJ, Holdrinet RSG, Geraedts JPM, Hagemeijer A, Van Der Blij-Philipsen M: Translocation 1;7 in dyshematopoiesis: Possibly induced with a nonrandom geographic distribution. Cancer Genet Cytogenet 12:283, 1984

25. Hutter JJ Jr, Hecht F, Kaiser-McCaw B, Hays T, Baranko P, Cohen J, Durie B: Bone marrow monosomy 7: Hematologic and clinical manifestations in childhood and adolescence. Hematol Oncol 2:5, 1984

26. Van Den Berghe H, Vermaelen K, Mercucci C, Barbieri D, Tricot G: The 5q- Anomaly. Cancer Genet Cytogenet 17:189, 1985

27. Dewald GW, Davis MP, Pierre RV, O'Fallon JR, Hoagland HC: Clinical characteristics and prognosis of 50 patients with a myeloproliferative syndrome and deletion of part of the long arm of chromosome 5. Blood 66:189, 1985

28. Yunis JJ, Brunning RD, Howe RB, Lobell M: High-resolution chromosomes as an independent prognostic indicator in adult acute nonlymphocytic leukemia. N Engl J Med 311:812, 1984

29. Rowley JD, Golomb HM, Vardiman JW: Nonrandom chromosome abnormalities in acute leukemic and dysmyelopoietic syndromes in patients with previously treated malignant disease. Blood 58:759, 1981

30. Groupe Français de Cytogénétique Hématologique: Chromosome analysis of 63 cases of secondary nonlymphoid blood disorders: A cooperative group. Cancer Genet Cytogenet 12:95, 1984

31. Michels SD, McKenna RW, Arthur DC, Brunning RD: Therapy-related acute myeloid leukemia and myelodysplastic syndrome: A clinical and morphologic study of 65 cases. Blood 85:1364, 1985.

Purified Plasma Factor XIIa Aggregates Human Neutrophils and Causes Degranulation

By Yanina T. Wachtfogel, Robin A. Pixley, Umberto Kucich, William Abrams, George Weinbaum,
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Plasma kallikrein has been shown to aggregate human neutrophils and release human neutrophil elastase. However, neutrophils resuspended in factor XII-deficient plasma released only 30% of the elastase compared with normal plasma. Isolated human neutrophils were aggregated in a concentration-dependent fashion by 0.06 to 0.6 U/mL factor XIIa (0.022 to 0.22 μmol/L). Factor XIIa (0.1 to 1.0 U/mL) also induced neutrophil degranulation as evidenced by a concentration-dependent release of the specific granule protein, lactoferrin, and azurophilic granule protease, elastase. The release of neutrophil elastase was biphasic, reaching 40% of maximum at 15 seconds with maximal release by 90 minutes. The active site of factor XIIa was required, since the synthetic inhibitor, D-Pro-Phe-Arg-CH_2Cl, which reacts with an essential histidine, and the natural plasma inhibitor, C1-inhibitor, which interacts with the critical serine, both inhibit by more than 90% the release of elastase. The heavy chain is also required, since factor XII fragments failed to aggregate neutrophils or stimulate degranulation. Factor XIIa (0.6 U/mL) can completely correct the defect in elastase release evident in factor XII-deficient plasma. These studies demonstrate that factor XIIa, at concentrations potentially obtainable in plasma in disease states, can activate neutrophils, and thus may participate in the inflammatory response.

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ENDOTHELIAL CELL INJURY may initiate the reactions in both the blood coagulation system[1] and recruitment of leukocytes[2] by exposing the subendothelial basement membrane. Neutrophil activation may occur by a variety of soluble agonists, such as the bacterial polypeptide *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine,[3] the plasma chemotaxin C5a,[4] or the secretagogue phorbol myristate acetate.[5] Human plasma kallikrein, an enzyme that appears during contact activation,[6] induces neutrophil chemotaxis[7] and stimulates them to undergo aerobic glycolysis,[8] aggregation,[9] and elastase release.[10] Neutrophils resuspended in normal plasma release human neutrophil elastase (HNE)[1] during blood coagulation by way of a thrombin-independent mechanism.[11] However, neutrophils resuspended in plasma deficient in prekallikrein or factor XII only release 30% of the amount of HNE that is released when the neutrophils are resuspended in normal plasma.[10]

In this study, we have demonstrated that in an in vitro plasma-free system, factor XIIa, consisting of a heavy polypeptide chain molecular weight (mol wt) 50,000 and a light chain mol wt 32,000 containing the active catalytic site,[12] can aggregate neutrophils and cause the release of lactoferrin and elastase. Furthermore, we have evaluated the contribution of the heavy chain and the catalytic portion of the factor XII molecule toward HNE release in the plasmatic milieu.

MATERIALS AND METHODS

Soybean trypsin inhibitor, *N*-formyl-L-Norleucyl-L-Leucyl-L-Phenylalanine (FNLP), Methoxy-succinyl-Ala-Ala-Pro-Val-pNA (MSAAPV), Triton X-100, and cytochalasin B were obtained from Sigma Chemical Co, St Louis. Flat-bottomed polystyrene microtiter plates (96 wells, Immulon No. 2) were obtained from either Fisher Scientific, King of Prussia, Pa, or Dynatech Laboratories, Alexandria, Va. Goat antirabbit IgG (heavy and light chains) covalently coupled to biotin and peroxidase-conjugated avidin were obtained from Cappel Laboratories, West Chester, Pa. Hanks' balanced salt solution (HBSS) was obtained from GIBCO Laboratories, Grand Island, NY. D-Pro-Phe-Arg-CH_2Cl was a generous gift from Dr Elliot Shaw, Friedrich Miescher Institute, Basle, Switzerland. Purified human lactoferrin and rabbit antihuman lactoferrin were obtained from Calbiochem-Behring Corp, La Jolla, Calif. Dextran, mol wt 100,000 to 200,000, was obtained from ICN, Cleveland.

Plasma. Factor XII-deficient plasma was kindly supplied by Dr Margaret Johnson, Wilmington Medical Center, Wilmington, Del. Prekallikrein-deficient plasma was generously provided by Dr Charles Abildgaard, University of California, Davis, Calif. After obtaining informed consent, venous blood from normal volunteers was collected directly into plastic syringes containing 1 vol trisodium citrate (final concentration 11 mmol/L) or acid-citrate-dextrose for each 9 vol of blood. No experimental differences were noted between these anticoagulants. Anticoagulated blood was centrifuged at 500 g for 15 minutes, to remove most cells, and the supernatant plasma at 27,000 g for 15 minutes at 4 °C, to prepare cell-free plasma.

Purified blood proteins. Factor XII was purified and activated to factor XIIa, as previously described by this laboratory.[12] Factor XII was a single protein component of mol wt 80,000 on both nonreduced and reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Factor XIIa was a single protein component of mol wt 80,000 on nonreduced gels and exhibited two chains of mol wt 52,000 and 28,000 upon reduction. The specific activity of factor XII was determined to be 62 U/mg. The concentration of factor XII in 1 mL of pooled normal plasma was defined as 1 U/mL and is equivalent to 0.37 μmol/L.[13] Factor XII fragments were purified as previously described[14] and appeared as a doublet of mol wt 30,000 and 28,000 on reduced gels.

Kallikrein was prepared by activation of purified prekallikrein by factor XII fragments.[15] On nonreduced SDS-PAGE, kallikrein was a doublet of mol wt 88,000 and 85,000, and on reduced gels, a heavy

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chain of mol wt 52,000 and two light chains of mol wt 36,000 and 33,000. Cl-inhibitor was purified as described[16] and had a major protein component at mol wt 105,000 and a minor component at 95,000. HNE was purified to homogeneity as described.[17] Antibody raised in rabbits to HNE produced one precipitin arc, either by immunodiffusion or immunoelectrophoresis against either purified HNE or a crude neutrophil extract. For enzyme-linked immunosorbent assays (ELISA), a dialyzed 35% ammonium sulfate-enriched IgG fraction was used.

Neutrophil isolation. Human neutrophils were isolated by anticoagulating 45 mL of venous blood with 5 mL of a solution containing 2.5% trisodium citrate, 1.4% citric acid monohydrate, 2% dextrose, 15% dextran, 0.15 mol/L sodium chloride, and 10 mmol/L adenosine diphosphate. Adenosine diphosphate was used to aggregate, and thus remove, any contaminating platelets. The neutrophils were then allowed to sediment at 1 g, followed by a single-step density separation using Ficoll-Hypaque,[18,19] ammonium chloride lysis of residual erythrocytes,[20] and extensive washes with HBSS. From 1 unit of whole blood, ~8 × 10^8 neutrophils of >98% purity and viability were isolated.

Neutrophil aggregation. Aggregometry was performed with modifications of the previously described method.[9] Ten million neutrophils were resuspended in a total volume of 1 mL HBSS containing a final concentration of 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$, pH 7.4, in the presence of various concentrations of factor XIIa. No cytochalasin B was added. Aggregation was measured with a platelet aggregometer (Chrono-log Corp, Havertown, Pa) using siliconized glass cuvettes containing siliconized stir bars revolving at 900 rpm.

Lactoferrin release in an in vitro plasma-free system. Ten million neutrophils were incubated without stirring at 37 °C in 1 mL of HBSS containing a final concentration of 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$, in the presence of various concentrations of factor XIIa. After 20 minutes, incubation mixtures were centrifuged at 13,000 g for five minutes at 23 °C in a Micro-Centrifuge (model 235A; Fisher Scientific Co, Pittsburgh). The supernatant solutions were recovered and frozen at −70 °C until lactoferrin was measured using an indirect competitive ELISA (see later).

HNE release in an in vitro plasma-free system. Ten million neutrophils were incubated without stirring at 37 °C in 1 mL of HBSS containing a final concentration of 2 mmol/L $CaCl_2$, 1.5 mmol/L $MgCl_2$, and 5 μg cytochalasin B.[21] After 20 minutes of preincubation, various agonists were added, the cells incubated for an additional 20 minutes, and then centrifuged and stored as described earlier. The supernatant fluids were recovered and frozen at −70 °C until HNE amidolytic activity was measured. HNE was measured using the chromogenic substrate MSAAPV (final concentration 1 mmol/L) as described.[22] Random samples were analyzed using an indirect competitive ELISA for HNE to confirm that the substrate MSAAPV was measuring HNE in our system (see later). It is important to note that factor XII, factor XIIa, or factor XII fragments, at concentrations used in these experiments, did not hydrolyze the substrate MSAAPV.

Neutrophil degranulation in an in vitro plasma system. Neutrophils (1 × 10^7/mL), in polypropylene tubes, were resuspended without stirring at 23 °C in normal plasma, factor XII-deficient plasma, or factor XII-deficient plasma reconstituted with 1 U/mL purified factor XIIa. No cytochalasin B was added. $CaCl_2$ (final concentration 30 mmol/L) was added, and after incubation for five or 60 minutes samples were centrifuged and the supernatant plasma stored as described, until HNE was measured immunochemically. The presence of α_1-antiprotease, the major plasma inhibitor of HNE, makes amidolytic activity measurements impractical in plasma.

ELISA for lactoferrin. An indirect competitive ELISA, modeled after the previously described procedure for HNE,[10] was used to measure lactoferrin. Serial dilutions of purified human lactoferrin in phosphate-buffered saline–0.05% Tween-20, pH 7.3 (PBS–T20) were used to generate a standard curve, or experimentally diluted samples were incubated with a limiting amount of rabbit antilactoferrin in siliconized tubes overnight at 4 °C. Flat-bottomed 96-well microtiter plates were coated by incubation with 200 μL of lactoferrin (100 ng/mL) in 0.1 mol/L carbonate buffer, pH 9.6, overnight at 4 °C. Antigen coating solution was removed from the plates by washing each well with PBS–T20 three times. The specific antiserum–lactoferrin reaction mixture was transferred in triplicate to wells on the plate, incubated for 60 minutes at 23 °C, the plate washed, the wells filled with a suitable dilution in PBS–T20 of goat antirabbit IgG conjugated to the indicator enzyme peroxidase, incubated 60 minutes at 23 °C, the plate washed and substrate for peroxidase added (100 mg/mL *O*-phenylenediamine, 0.1 mol/L citrate buffer, pH 4.5, 0.06% H_2O_2). After 30 minutes the absorbance at 450 nm was measured in a microtiter plate reader (BioTek model EL307, Burlington, Vt). The absorbance of the standards was plotted against the log of their lactoferrin concentration and the data fit by linear regression, using a microcomputer. The concentration of experimental samples was derived from the best fit line of the standard curve. Figure 1 demonstrates that increasing amounts of antigen were inversely proportional to the absorbance obtained, with the useful range of this assay being 1 to 20 ng/mL lactoferrin.

ELISA for HNE. Immunochemical measurement of total HNE concentration was performed by an indirect competitive ELISA in 96-well microtiter plates as previously described[10,23] with the following modifications. Goat antirabbit IgG, conjugated with biotin, was added to each plate and incubated for 60 minutes at 23 °C, the plates washed, and peroxidase-avidin added for 30 minutes at 23 °C. The plates were again washed, substrate added, incubated for 40 to 60 minutes, and the absorbance was measured at 450 nm.

Lactate dehydrogenase assay. Release of the cytoplasmic enzyme lactate dehydrogenase was monitored after incubation on supernatant aliquots and Triton X-100 (0.5%) extracts of neutrophils by the method of Reeves and Fimognari,[24] to determine whether neutrophil lysis had occurred during agonist exposure.

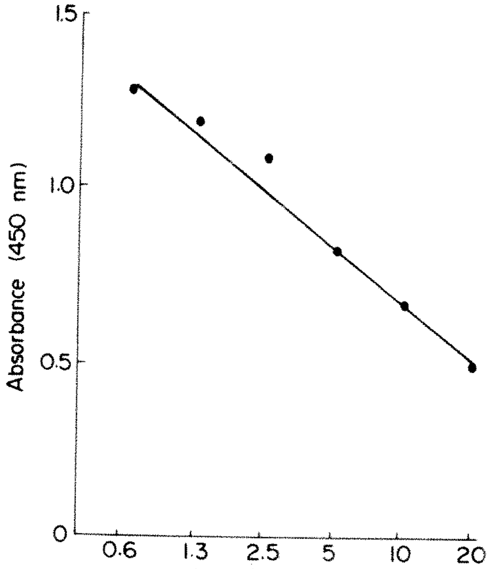

Fig 1. Competitive ELISA for lactoferrin. The ELISA was performed as described in Materials and Methods. A standard curve was constructed using various concentrations of lactoferrin, and the resultant absorbance plotted on the ordinate. All values are a mean of at least three determinations.

RESULTS

Aggregation of human neutrophils by factor XIIa. Purified human factor XIIa, 0.3 U/mL (0.11 μmol/L), incubated with neutrophils in HBSS, containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ (final concentration), induced full aggregation (Fig 2). The initial rate of aggregation was proportional to the concentration of the agonist factor XIIa over the concentration range 0.06 to 0.6 U/mL (0.02 to 0.22 μmol/L), reaching a plateau at 0.3 U/mL (0.11 μmol/L) (Fig 2 inset). The aggregation was always biphasic in response to factor XIIa. The initial rate of aggregation was calculated and expressed as change in transmittance per minute. Under the same conditions, zymogen factor XII (0.37 μmol/L), factor XII fragments (0.37 μmol/L), and factor XIIa, in the absence of exogenous calcium, failed to aggregate neutrophils.

Human neutrophil degranulation by factor XIIa. Purified human factor XIIa, 0.2 to 1.0 U/mL (0.074 to 0.37 μmol/L), induced neutrophils to release 0.78 ± 0.25 to 2.85 ± 1.07 μg/mL lactoferrin (8% ± 3% to 31% ± 12% of the total lactoferrin per 10^7 neutrophils) in a concentration-dependent manner (Fig 3). The release of lactoferrin was determined by a competitive ELISA for lactoferrin.

Factor XIIa, 0.1 to 1.0 U/mL (0.037 to 0.37 μmol/L) induced neutrophils, primed with cytochalasin B (5 μg/mL), to release 0.35 ± 0.12 to 1.92 ± 0.75 μg/mL HNE (5% ± 2% to 27% ± 10% of the total HNE per 107 neutrophils) in a concentration-dependent manner. The release of the enzyme was determined by an activity assay for HNE, using the amidolytic substrate (MSAAPV) (Fig 4). This release was not due to cell lysis, since <2% of the total cellular lactate dehydrogenase solubulized by 0.5% Triton X-100 appeared in the incubation medium (data not shown).

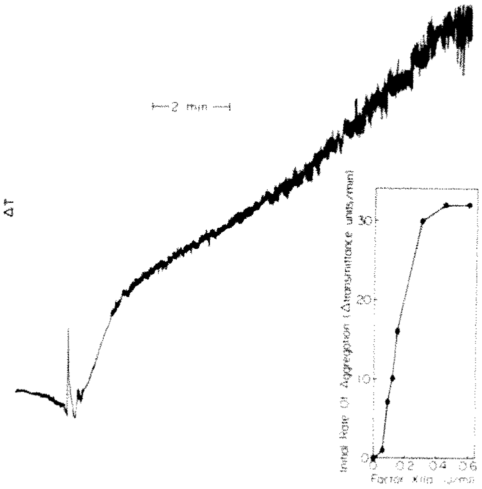

Fig 2. Aggregation of neutrophils stimulated by factor XIIa. Neutrophils (1 × 10^7/mL) were incubated in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ in a Chrono-log aggregometer at 37 °C, with various concentrations of factor XIIa. This is an actual neutrophil aggregation tracing in response to factor XIIa (0.3 U/mL). The spike is the result of addition of factor XIIa. The graph inset presents data at multiple concentrations of factor XIIa. The initial rates of aggregation are calculated from the slope of each tracing and expressed as maximal change per unit time. Each point is one of three studies at that concentration showing similar results.

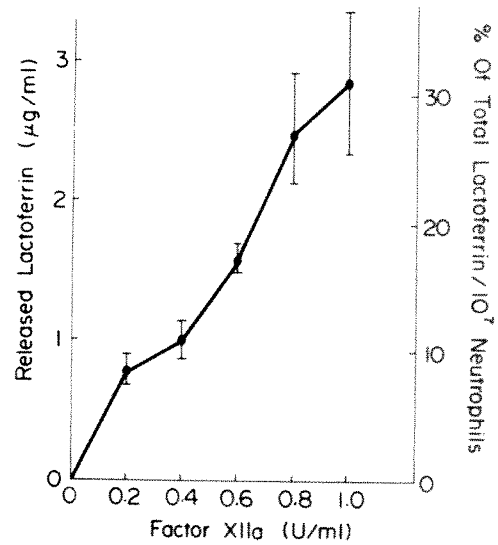

Fig 3. Lactoferrin release stimulated by factor XIIa. Neutrophils (1 × 10^7/mL) in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ were incubated at 37 °C for 20 minutes in the presence of increasing concentrations of factor XIIa. Samples were centrifuged at 13,000 g for five minutes at 23 °C and lactoferrin was measured in these samples, using an indirect competitive ELISA for lactoferrin. Results are expressed as released lactoferrin (μg/mL), as well as the percentage of total lactoferrin per 10^7 neutrophils. These data represent the mean of two determinations.

Zymogen factor XII (0.37 μmol/L), factor XIIa (0.37 μmol/L) incubated with D-Pro-Phe-Arg-CH_2Cl (5 μmol/L) or $\bar{C1}$-inhibitor (1.5 μmol/L), Factor XII fragments, or calcium alone failed to initiate release of HNE. Factor XIIa (0.37 μmol/L) caused the release of 2.36 ± 0.85 μg/mL HNE, similar to the 2.47 ± 0.35 μg/mL HNE released by the chemotactic peptide FNLP (100 nmol/L) (Table 1). The incubation of D-Pro-Phe-Arg-CH_2Cl (5 μmol/L) or $\bar{C1}$-inhibitor (1.5 μmol/L) with FNLP (100 nmol/L) had no inhibitory effect on the ability of FNLP to induce neutrophil degranulation. No HNE was detected on stimulation of neutrophils by factor XIIa (0.37 μmol/L) in the absence of cytochalasin B.

To assess the kinetics of HNE release, purified human plasma factor XIIa, 0.75 U/mL (0.28 μmol/L) was incubated with neutrophils that had been primed at 37 °C with cytochalasin B, in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$. At various times, an aliquot was removed and processed. Within the first 15 seconds HNE was released from the neutrophils, amounting to 40% of the maximum that could be released by factor XIIa. A slower release of HNE followed, which reached a plateau by 90 minutes (Fig 5).

Degranulation of human neutrophils in plasma. In an in vitro plasma system, when neutrophils (1 × 10^7) were incubated at 23 °C for 60 minutes in 1 mL of normal human plasma in the presence of 30 mmol/L $CaCl_2$, 2.8 μg HNE was released, as measured immunochemically (Table 2). However, neutrophils resuspended in factor XII-deficient

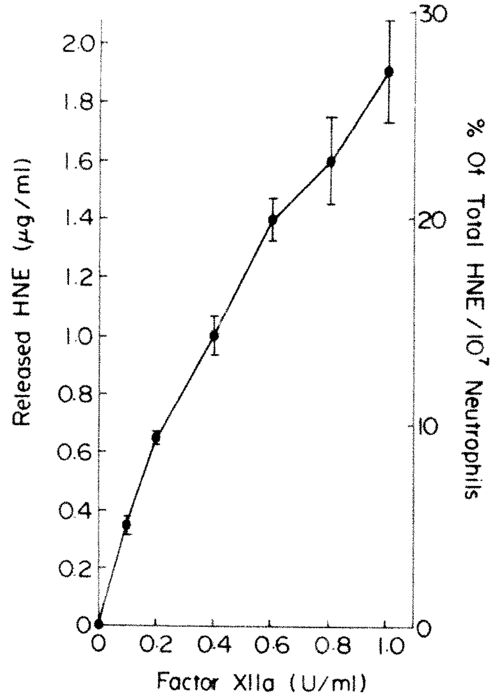

Fig. 4. HNE release stimulated by factor XIIa. Neutrophils (1×10^7/mL) in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ were preincubated with cytochalasin B (5 μg) at 37 °C for 20 minutes. Increasing concentrations of factor XIIa were then added to the cells and incubation continued for an additional 20 minutes. Samples were centrifuged at 13,000 g for five minutes at 23 °C, and the HNE activity was measured in these supernatants, using the amidolytic substrate MSAAPV. Results are expressed as released HNE (μg/mL), as well as the percentage of total HNE per 10^7 neutrophils. These data represent mean ± SEM of three determinations.

plasma released only 1.0 μg HNE. Neutrophils incubated with factor XII-deficient plasma that was reconstituted with purified factor XIIa released 3.0 μg HNE. Neutrophils resuspended in normal human plasma for only five minutes under the same conditions released 0.7 μg HNE, whereas neutrophils resuspended in factor XII-deficient plasma released 0.1 μg HNE. Upon incubation of neutrophils in factor XII-deficient plasma reconstituted with factor XIIa, 1.3 μg HNE was released.

DISCUSSION

Evidence suggests that the plasma contact factor, factor XIIa, is able to stimulate neutrophils to aggregate (Fig 2) and degranulate (Figs 3 and 4) in an in vitro plasma-free system. These observations are similar to those observed for plasma kallikrein.[9,10,25] Zymogen factor XII, as well as factor XIIa, inhibited by the active site inactivator D-Pro-Phe-Arg-CH_2Cl, which reacts with an essential histidine, or by $\bar{C}1$-inhibitor, the major inhibitor of activated factor XII plasma, which reacts with the light chain serine,[26,27] failed to elicit any neutrophil degranulation (Table 1). The observations indicate the requirement for the catalytic site of the enzyme factor XIIa. Factor XII fragments, similar to the light chain and containing the catalytic site but not the heavy

Table 1. Effect of Agonist on HNE Release From Neutrophils

Agonist	HNE Released (μg/mL)
Human plasma factor XIIa (0.37 μmol/L)	2.36 ± 0.85
Human plasma factor XII (0.37 μmol/L)	0.01 ± 0.02
Human plasma factor XII fragments (0.37 μmol/L)	0.03 ± 0.05
Human plasma factor XIIa (0.37 μmol/L) + D-Pro-Phe-Arg-CH_2Cl (5 μmol/L)	0.26 + 0.21
Human plasma factor XIIa (0.37 μmol/L) + C1-inhibitor (1.5 μmol/L)	0.18 ± 0.18
FNLP (100 nmol/L)	2.47 ± 0.35
FNLP (100 nmol/L) + D-Pro-Phe-Arg-CH_2Cl (5 μmol/L)	2.50 ± 0.06
FNLP (100 nmol/L + C1-inhibitor (1.5 μmol/L)	2.23 ± 0.24
No calcium added	0.00 ± 0.00

Neutrophils (1×10^7/mL) in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ were preincubated with cytochalasin B (5 μg) for 20 minutes at 37 °C. An agonist was added and incubation continued for an additional 20 minutes. The supernatants were harvested by centrifugation at 13,000 g for five minutes and were assayed for HNE, using the substrate MSAAPV. Amidolytic activities were confirmed with immunochemical measurement of HNE. No HNE release was observed in the absence of calcium. FNLP, the chemotactic peptide, with its methionine replaced by a Norleucyl, was used in these studies to obviate the possible oxidation of the methionine. Results are a mean ± SEM of three experiments.

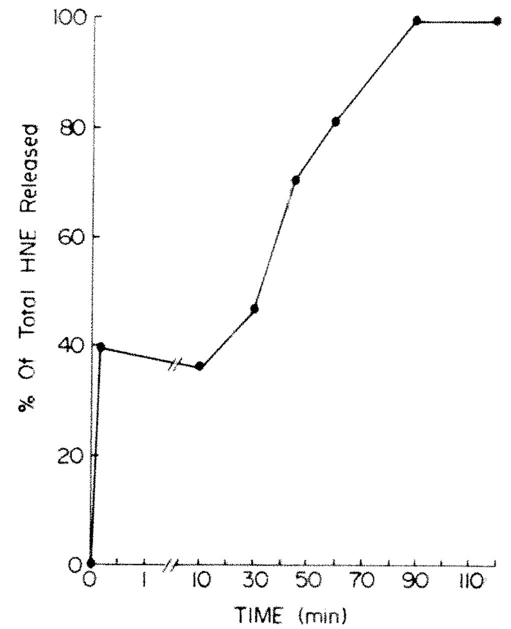

Fig 5. Kinetics of neutrophil degranulation in response to factor XIIa. Neutrophils (1×10^7/mL) in HBSS containing 2 mmol/L $CaCl_2$ and 1.5 mmol/L $MgCl_2$ were preincubated with cytochalasin B (5 μg) at 37 °C for 20 minutes. Factor XIIa (0.75 U/mL) was incubated with the cells for various time intervals, after which samples were centrifuged at 13,000 g for five minutes, and the HNE activity was measured in these supernatants, using the amidolytic substrate MSAAPV. These data represent the mean of two determinations.

Table 2. Effect of Factor XIIa in Plasma on Neutrophil Release of HNE

	5 min, HNE Released at μg/mL (Percentage of HNE Release Compared With Normal Human Plasma)	60 min, HNE Released at μg/mL (Percentage HNE Release Compared With Normal Human Plasma)
Normal human plasma	0.7 (25)	2.8 (100)
Human factor XII-deficient plasma	0.1 (4)	1.0 (39)
Human factor XII-deficient plasma + 0.6 U/mL factor XIIa	1.3 (46)	3.0 (107)

Neutrophils (1 × 10^7) were resuspended in 1 mL of normal human plasma, factor XII-deficient plasma, or factor XII-deficient plasma reconstituted with purified factor XIIa, all of which contained 30 mmol/L $CaCl_2$. Incubations were done in polypropylene tubes at 23 °C for five or 60 minutes, followed by centrifugation at 13,000 g for five minutes. Supernatants were assayed for HNE using an indirect competitive ELISA. Results are a mean of two separate experiments, each assayed in triplicate.

chain, also failed to activate neutrophils, indicating the participation of the heavy chain in this reaction.

This interaction of neutrophils with various molecular forms of activated factor XII parallels the cell interaction with the different forms of kallikrein. The active form of the enzyme factor XIIa, like α-kallikrein, which contains the unmodified heavy chain as well as the active site,[14,25] induces both neutrophil aggregation[9] and degranulation.[10] The zymogens factor XII and prekallikrein, as well as the active enzymes α-kallikrein or factor XIIa, which have been inactivated, failed to elicit any neutrophil activation.[9,10] The most striking parallel is the finding that both active plasma contact enzymes, factor XII fragments or β-kallikrein (which do not contain their respective intact heavy chains),[14] failed to induce neutrophil degranulation.[25] Therefore, we find the requirement of an active site and intact heavy chain in both of these plasma contact factors for their stimulation of neutrophils.

The kinetics of HNE release from neutrophils exposed to factor XIIa revealed that 40% of the maximum HNE was released within the first 15 seconds (Fig 5), which is similar to that observed after stimulation by kallikrein[10] or the release of myeloperoxidase by C5a.[28] This rapid release of HNE was not associated with leakage of cytoplasmic lactate dehydrogenase into the medium, indicating that this degranulation was due to granule exocytosis induced by factor XIIa and not by cell death.

In our in vitro plasma-free system, to demonstrate HNE (azurophilic granule) release, it was necessary to prime the neutrophils with cytochalasin B, an inhibitor of microtubules. Biochemical and electron microscopic studies of neutrophils indicate that exposure to cytochalasin B allows the phagosomes to fuse with one another and the plasma membrane, priming the extrusion of enzymes extracellularly that usually are involved in intracellular digestion within these phagosomes.[29] The identity of the additional component in plasma supporting the release of HNE in a system free of cytochalasin B is still under investigation. It should be noted that release of the specific granule protein lactoferrin occurred in the absence of cytochalasin B, as reported for other neutrophil agonists.

Plow[11] has shown that in an in vitro system neutrophils release HNE during blood coagulation by way of a thrombin-independent mechanism. This observation was confirmed and extended to show that the neutrophils resuspended in prekallikrein-deficient plasma or factor XII-deficient plasma release only 25% or 39%, respectively, of the HNE released in normal plasma.[10] The conclusion from those earlier experiments was that decreased HNE release in factor XII-deficient plasma was due to its requirement for the activation of kallikrein. In view of this study in an in vitro plasma-free system, however, a more attractive possibility appeared to be that factor XIIa was contributing to the release of HNE from neutrophils. This hypothesis was tested by reconstituting factor XII-deficient plasma with purified factor XIIa. We found that at 60 minutes factor XIIa completely corrected the defect in the HNE release observed in factor XII-deficient plasma, indicating that factor XIIa either directly stimulates neutrophils or causes the formation of an agonist, requiring the presence of factor XII. The observation that at five minutes, factor XIIa in factor XII-deficient plasma releases *more* HNE than is released in normal plasma at five minutes indicates that factor XIIa formation is rate limiting in this system. This conclusion is in agreement with the ability of factor XIIa to release 40% of HNE in 15 seconds in a purified system. Furthermore, the nature of the surface of the test tube is critical, since, at five minutes, in normal plasma exposed to a polypropylene surface, only 31% of HNE was released, while in a glass tube, 100% was released.[10] As might be expected, contact activation is central in the generation of the neutrophil agonist.

Two enzymes, kallikrein and factor XIIa, activated by surface exposure are capable of stimulating neutrophils. Factor XIIa can convert factor XI to XIa, but factor XIa enzyme does not stimulate neutrophils[9] and no abnormality occurs in factor XI-deficient plasma,[10] ruling out later coagulation proteases. Both factor XIIa and kallikrein serve as weak activators of plasminogen,[30,31] but plasmin does not stimulate neutrophils directly.[9] C5a is a well-known agonist for neutrophils, but our previous study shows that plasma deficient in C5 shows no defect in surface-mediated HNE release.[10] Although kallikrein can convert prorenin to renin, and thus form angiotensin II, this reaction does not proceed in normal plasma at neutral pH, and therefore, neither renin nor the peptide that might be potential candidates for agonists would be present.[32] Finally, kallikrein and factor XIIa can release bradykinin from kininogen in plasma, but bradykinin does not induce neutrophil aggregation.[9] Thus it appears that kallikrein or factor XIIa or both are likely to be the agonists formed in this in vitro plasma system under study.

The inability of either factor XII-deficient plasma or prekallikrein-deficient plasma to induce neutrophils to release large amounts of HNE may be related to the findings of Rebuck,[33] using the skin window to assess in vivo the chemotaxis of leukocytes in response to tissue or microvascular injury. After six hours of the inflammatory stimulus in a volunteer with congenital factor XII deficiency, only 4% of the neutrophils are attracted to the site, whereas in an individual deficient in prekallikrein, only 20% of the leukocytes are attracted to the site as compared with that of a normal subject.

Neutrophils resuspended in plasma from patients with hereditary angioedema, exhibiting a deficiency in C$\bar{1}$-inhibitor,[34] release 2.3-fold more HNE than neutrophils resuspended in normal plasma.[10] This finding is especially important when considering the potential role of factor XIIa in the activation of neutrophils in these patients, since C$\bar{1}$-inhibitor accounts for more than 90% of the total plasma inhibitory capacity toward factor XIIa.[12] In contrast, plasma kallikrein is inhibited by both C$\bar{1}$-inhibitor and α_2-macroglobulin. Therefore, in plasma from a patient with hereditary angioedema, α_2-macroglobulin may still partially inhibit kallikrein, whereas factor XIIa, unopposed, may contribute to the infirmity of these patients by way of its ability to initiate neutrophil degranulation. Although factor XII fragments do not directly activate neutrophils, they can initiate the classic pathway of the complement system by activating the C1 macromolecule,[35,36] providing the well-known stimulus for neutrophils, C5a.[4]

The observation that factor XIIa is an agonist of neutrophils is not surprising, since the heavy chain of the molecule has recently been found to contain homologous domains to other biologically active proteins, including two growth factor domains.[37] The presence of growth factor domains suggests the possibility that binding of the agonist to the cell may occur, with resultant aggregation and degranulation. This mechanism has been proposed in the activation of neutrophils induced by platelet-derived growth factor.[38] The putative binding of factor XIIa to neutrophils would also provide a mechanism for the enzyme to activate neutrophils even in the presence of plasma C$\bar{1}$-inhibitor.

A wide variety of diseases has been reported to be associated with activation of the contact system, including gram-negative hypotensive septicemia,[39] hyperacute renal allograft rejection,[40] typhoid fever,[41] and nephrotic syndrome.[42,43] These conditions were associated with decrease in factor XII to levels ranging from 15% to 40% of normal, presumably due to conversion to an activated enzymatic form. The resulting factor XIIa concentrations of 60% to 80% of maximum activatable zymogen would release substantial amounts of HNE (Fig 4).

The activation of neutrophils with subsequent release of HNE, under pathophysiologic conditions in which factor XIIa may be formed, may play a role in endothelial cell injury. HNE has been shown to release nucleotides from endothelial cells,[44] which are indicators of cell injury. Endothelial cells respond to stimuli by synthesizing PGI_2, which is a potent vasodilator. The loss of endothelium-dependent vasodilation may result in unopposed vasoconstrictor responses, which might contribute to the paradoxical vasospasm seen in some conditions of peripheral vascular disease, such as those associated with Raynaud's phenomenon. Purified HNE has been shown to produce morphological destruction of lung parenchyma.[45] In addition, HNE has been implicated in the cleavage of plasma contact factors,[46] thus serving as a possible positive feedback mechanism for subsequent amplification of neutrophil activation and degranulation.

REFERENCES

1. Harpel PC: Studies on the interaction between collagen and a plasma kallikrein-like activity. Evidence for a surface-active system. J Clin Invest 51:1813, 1972

2. Harlan JM, Killen PD, Harker LA, Striker GE, Wright DG: Neutrophil-mediated endothelial injury *in vitro*. J Clin Invest 68:1394, 1981

3. Fletcher MP, Gallin JI: Degranulating stimuli increase the availability of receptors on human neutrophils for the chemoattractant F-Met-Leu-Phe. J Immunol 124:1585, 1980

4. Wright DG: The neutrophil as a secretory organ of host defense, in Gallin JI, Fauci AS (eds): Advances in Host Defense Mechanisms, vol 1. New York, Raven, 1982, p 75

5. Goldstein IM, Hoffstein ST, Weissmann G: Mechanisms of lysosomal enzyme release from human polymorphonuclear leukocytes. Effects of phorbol myristate acetate. J Cell Biol 66:647, 1975

6. Margolis J: Activation of plasma contact with glass. Evidence for a common reaction which releases plasma kinin and initiates coagulation. J Physiol 44:1, 1959

7. Kaplan AP, Kay AB, Austen KF: A prealbumin activator of prekallikrein. III. Appearance of chemotactic activity for human neutrophils by the conversion of human prekallikrein to kallikrein. J Exp Med 135:81, 1972

8. Goetzl EJ, Austen KF: Stimulation of human neutrophil aerobic glucose metabolism by purified chemotactic factors. J Clin Invest 53:591, 1974

9. Schapira M, Despland E, Scott C, Boxer LA, Colman RW: Purified human plasma kallikrein aggregates human blood neutrophils. J Clin Invest 69:1191, 1982

10. Wachtfogel YT, Kucich U, James HJ, Scott CF, Schapira M, Zimmerman M, Cohen AB, Colman RW: Human plasma kallikrein releases neutrophil elastase during blood coagulation. J Clin Invest 72:1672, 1983

11. Plow E: Leukocyte elastase release during blood coagulation. A potential mechanism for activation of the alternative fibrinolytic pathway. J Clin Invest 69:564, 1982

12. Pixley RA, Schapira M, Colman RW: The regulation of factor XIIa by plasma proteinase inhibitors. J Biol Chem 260:1723, 1985

13. Tankersley DL, Alving BM, Finlayson JS: Preparation of β-XIIa (Hageman factor fragment) from human plasma. Thromb Res 25:307, 1982

14. Revak SD, Cochrane CG, Johnson AR, Hugli TE: Structural changes accompanying enzymatic activation of human Hageman Factor. J Clin Invest 54:619, 1974

15. Scott CF, Liu CY, Colman RW: Human plasma prekallikrein: A rapid high yield method for purification. Eur J Biochem 100:77, 1981

16. Reboul A, Arlaud GF, Sim RB, Colomb MG: A simplified procedure for the purification of C$\bar{1}$-inactivator from human plasma.

Interaction with complement subcomponents Clr and Cls. FEBS Lett 79:45, 1977

17. Baugh RJ, Travis J: Human leukocyte granule elastase: Rapid isolation and characterization. Biochemistry 15:836, 1976

18. Oseas RS, Boxer LA, Butterick C, Baehner RL: Differences in polymorphonuclear leukocyte aggregating responses among several species in response to chemotactic stimulation. J Lab Clin Med 96:213, 1980

19. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest 21:77, 1968 (suppl 96)

20. Agostoni A, Ideo G: Separation of large numbers of lymphocytes from human blood. Experientia 21:82, 1965

21. Goldstein I, Hoffstein S, Gallin J, Weissman G: Mechanisms of lysosomal enzyme release from human leukocytes: Microtubule assembly and membrane fusion induced by a component of complement. Proc Natl Acad Sci USA 70:2916, 1973

22. Nakajma K, Powers JC, Ashe BM, Zimmerman M: Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. J Biol Chem 254:4027, 1979

23. Kucich U, Abrams WR, James HL: Solid phase immunoassay of dog neutrophil elastase. Anal Biochem 109:403, 1980

24. Reeves WJ, Fimognari GM: An improved procedure for the preparation of crystalline lactic dehydrogenase from dog heart. J Biol Chem 238:3853, 1963

25. Colman RW, Wachtfogel YT, Kucich U, Weinbaum G, Hahn S, Pixley RA, Scott CF, deAgostini A, Burger D, Schapira M: Effect of cleavage of the chain of human plasma kallikrein on its functional properties. Blood 65:311, 1985

26. Forbes CO, Pensky J, Ratnoff OD: Inhibition of activated Hageman factor and activated plasma thromboplastin antecedent by purified Cl inactivator. J Lab Clin Med 76:809, 1970

27. Schreiber AD, Kaplan AP, Austen KF: Inhibition of Cl INH of Hageman factor fragment activation of coagulation, fibrinolysis, and kinin generation. J Clin Invest 52:1402, 1973

28. Sklar LA, McNeill VM, Jesaitis AJ, Painter RG, Cochrane CG: A continuous spectroscopic analysis of the kinetics of elastase secretion by neutrophils. J Biol Chem 257:5471, 1982

29. Zurier RB, Hoffstein S, Weissmann G: Cytochalasin B: Effect on lysosomal enzyme release from human leukocytes. Proc Natl Acad Sci USA 70:844, 1973

30. Goldsmith GH, Saito H, Ratnoff OD: The activation of plasminogen by Hageman (factor XII) and Hageman factor fragments. J Clin Invest 62:54, 1978

31. Colman RW: Activation of plasminogen by human plasma kallikrein. Biochem Biophys Res Commun 35:273, 1969

32. Purdon AD, Schapira M, deAgostini A, Colman RW: Plasma kallikrein and prorenin in patients with hereditary angioedema. J Lab Clin Med 105:694, 1985

33. Rebuck JW: The skin window as a monitor of leukocytic functions in contact activation factor deficiencies in man. Am J Clin Pathol 79:405, 1983

34. Donaldson VH, Evans RR: A biochemical abnormality in C'l-esterase. Am J Med 35:37, 1963

35. Donaldson VH: Mechanisms of activation of C'l-esterase in hereditary angioneurotic edema plasma *in vitro:* The role of Hageman factor, a clot promoting agent. J Exp Med 127:411, 1968

36. Ghebrehiwet B, Silverberg M, Kaplan AP: Activation of the classical pathway of complement by Hageman factor fragment. J Exp Med 153:665, 1981

37. McMullen BA, Fujikawa K: Amino acid sequence of the heavy chain of human α-Factor XIIa (activated Hageman Factor). J Biol Chem 260:5328, 1985

38. Williams LT, Antoniades HN, Goetzl EJ: Platelet-derived growth factor stimulates mouse 3T3 cell mitogenesis and leukocyte chemotaxis through different structural determinants. J Clin Invest 72:1759, 1983

39. Mason JW, Kleeberg UR, Dolan P, Colman RW: Plasma kallikrein and Hageman factor in gram-negative bacteremia. Ann Intern Med 73:545, 1970

40. Colman RW, Girey G, Galvanek EG, Busch GJ: Human renal allografts: The protective effects of heparin, kallikrein activation, and fibrinolysis during hyperacute rejection, in Kaulla KV (ed): Symposium on Coagulation Problems in Transplanted Organs. Springfield, Ill, Charles C. Thomas, p 87

41. Colman RW, Scott CF, Gilman RH, Edelman R: Plasma kallikrein activation and inhibition during typhoid fever. J Clin Invest 61:287, 1978

42. Honig GR, Lindley A: Deficiency of Hageman factor (factor XIII) in patients with the nephrotic syndrome. J Pediatr 78:633, 1971

43. Lange LG, Carvalho A, Bagdasarian A, Lahiri B, Colman RW: Activation of Hageman factor in the nephrotic syndrome. Am J Med 56:565, 1974

44. LeRoy EC, Ager A, Gordon JL: Effects of neutrophil elastase and other proteases on porcine aortic endothelial prostaglandin I_2 production, adenine nucleotide release, and responses to vasoactive agents. J Clin Invest 74:1003, 1984

45. Janoff A, Sloan B, Weinbaum G, Damiano V, Sandhaus RA, Elias J, Kimbel P: Experimental emphysema induced with purified human neutrophil elastase: Tissue localization of instilled protease. Am Rev Respir Dis 115:461, 1977

46. McGuire WW, Spragg RG, Cohen AB, Cochrane CG: Studies on the pathogenesis of the adult respiratory distress syndrome. J Clin Invest 69:543, 1982

Thrombin Causes Subsecond Changes in Protein Phosphorylation of Platelets

By D.J. Carty, F. Spielberg, and A.R.L. Gear

We have developed a general quenched-flow approach to study platelet function as early as 0.3 seconds after stimulation. Phosphorylation of 20- and 40-kd proteins has been analyzed during the first five seconds of platelet response to thrombin from 0.1 to 5.0 U/mL and compared with the progress of aggregation and serotonin secretion. The onset time for aggregation and phosphorylation of both proteins was less than one second, although with lowest (<0.5 U/mL) thrombin levels, a lag of up to 0.6 seconds occurred before 40K phosphorylation increased. The thrombin sensitivity of aggregation and 20K phosphorylation was approximately twice that of 40K phosphorylation, with Ka values of 0.51 and 0.53 *v* 1.10 U/mL, respectively. External calcium was necessary for maximal 20K phosphorylation, since EDTA inhibited this by 30%. The 40K phosphorylation was not affected by EDTA. Platelet activation by thrombin thus induced biochemical changes well before one second. The quenched-flow approach may help to reveal relationships between phospholipase activation, calcium fluxes, and protein phosphorylation during these early periods of platelet function.

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RECEPTOR-MEDIATED FLUXES of calcium are common to the stimulation of many cell types by agents such as neurotransmitters, peptide hormones, and secretogogues.[1] These calcium fluxes are usually accompanied by increased turnover of phosphatidylinositol, phosphorylation of specific proteins, and an intracellular response appropriate to the cell type.[2] For the blood platelet, stimulation by compounds such as thrombin causes changes in morphology, cell aggregation, and secretion of specific cytoplasmic contents.[3] The relationships of lipid turnover, protein phosphorylation, and ion movements in this stimulus–response sequence are not clear, partly because of the rapidity with which the changes occur.

We have developed a general quenched-flow approach[4] for studying early events in platelet function, including "shape change,"[5] aggregation,[4] and secretion.[6] This method allows analysis of platelet function and biochemical changes during the first five seconds of response to stimulation, a time scale approaching that suggested from theoretical calculations of the speed required for efficient hemostasis.[7,8] We have found that major morphological changes may be nearly complete within two seconds after platelet activation by adenosine diphosphate (ADP) or thrombin,[5] and that aggregation, as detected by single-particle counting, may be about 80% complete within five seconds.[4,6] The quenched-flow approach has now been used to follow the thrombin-stimulated phosphorylation of two platelet proteins (molecular weights [mol wt] 20 kd and 40 kd). The changes in phosphorylation of these proteins over time have been compared with the extent of aggregation, secretion, and involvement of external Ca^{++}. The influence of thrombin concentration (0.1 to 5 U/mL) has also been assessed. The data reveal a rapid phosphorylation (<1.0 second) of the 20K and 40K proteins, but with important differences in kinetics and sensitivity to thrombin and Ca^{++}. Conditions were also found in which marked phosphorylation occurred in the absence of aggregation or secretion. Some of these data have been presented in preliminary form.[9,10]

MATERIALS AND METHODS

Platelet preparation and labeling. Venous forearm blood (90 mL) was drawn from normal volunteers, anticoagulated with acid-citrate-dextrose (ACD) and platelet-rich plasma (PRP) prepared as described previously.[11]

The platelets were labeled by incubating 30 to 40 mL of PRP at 37 °C for 60 minutes with 10 to 20 mCi [^{32}P]-inorganic phosphate (New England Nuclear, Boston), or by preincubating 10 mL PRP for 40 minutes at 37 °C, adding 1.67 μCi (final concentration 7.26×10^{-9} mol/L) of 2-[^{14}C]-serotonin (Amersham, Arlington Heights, Ill), and incubating 20 minutes more. Extra ACD was added (1:10 final volume) to improve subsequent recovery and viability, and the PRP was then centrifuged for 15 minutes at 350 g. The platelets subsequently were suspended in ACD, centrifuged again, and finally resuspended in an Eagle's salt solution (GIBCO, Grand Island, NY) containing human fibrinogen at 0.50 or 0.75 mg/mL.[12] The labeled platelet-rich Eagle's (PRE) was gassed with a 5% CO_2 and 95% air mixture to preserve platelet function,[13] and aliquots of PRE (statics) were set aside to provide values for platelets not subject to quenched-flow.

Quenched-flow methods. Before any quenched-flow run, the PRE was incubated five minutes at 37 °C, sometimes in the presence of 2 mmol/L EDTA. Then, for release experiments, imipramine (4.2 μmol/L) was added to prevent reuptake of serotonin.[14] The PRE was placed in one syringe of the quenched-flow apparatus described previously.[4,6] The principle behind this system is to pump platelets through narrow-bore tubing (0.2 to 0.3 mm internal diameter), together with an inducing agent, and to quench the reaction at the outlet. A glutaraldehyde solution (3% vol/vol in 0.15 mol/L NaCl) was used as quencher for assessing aggregation efficiency and serotonin secretion.[6] Perchloric acid (3N in 0.15 mol/L NaCl) was used to quench the reacting platelets for subsequent protein electrophoresis procedures.

The speed of quenching was assessed in three ways. First, a "reverse-order" experiment was carried out in which glutaraldehyde was added before ADP in the quenched-flow device. Aggregation was normal when ADP was added first but was absent when glutaraldehyde was added first. Second, glutaraldehyde was used to stop the transformation of platelet shape from disks into pseudopodial forms.[5] Linear regression analysis of the data suggested a quenching time of −0.10 seconds. Third, trichloroacetic acid was used in an experiment measuring the formation of cyclic adenosine monophosphate (cAMP) in platelets stimulated by 1 μmol/L prostaglandin E_1 (PGE_1). Synthesis of cAMP proceeded linearly and a linear regression plot of time points up to five seconds could be traced back to the zero time control value at 0.13 seconds.

These three experimental approaches suggest that the speed of

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quenching in our reaction system occurs with a dead time of about 0.1 second. Therefore, the reaction times for protein phosphorylation to be described later may be assumed valid within 0.1 second.

Platelet aggregation. To evaluate the extent of platelet aggregation, 60 μL of the effluent from the quenched-flow apparatus were diluted to 10 mL with saline and analyzed in a resistive particle counter (Particle Data, Elmhurst, Ill). Aggregation is expressed as the loss of singlet platelets at various reaction times, with the preaggregation value serving as 100%.[4] In these experiments, the inducing agent was either saline (as a control), or 0.1, 0.5, 1, or 5 U/mL thrombin in saline. Because a concentration of 7 to 10 U/mL can develop in vivo,[15] the largest concentration is maximal but not outside physiologic limits.

Serotonin secretion. To evaluate $[^{14}C]$-serotonin secretion, the quenched PRE was centrifuged for five minutes at 2,000 g, yielding platelet-poor Eagle's supernatant (PPE). Then a 50-μL portion was counted in scintillation "cocktail" and compared to control PPE and PRE. Serotonin secretion is expressed as a percentage of the total $[^{14}C]$-serotonin contained in the platelets.

Protein phosphorylation. The ice-cold precipitates from 600 μL of reacting platelets quenched with perchloric acid were washed once with 1 mol/L NaCl at 0 °C, dissolved in 200 μL of a Tris buffer (0.0625 mol/L), pH 6.8, containing 2% sodium dodecyl sulfate (SDS), reduced with 2-mercaptoethanol and electrophoresed in 7% or 9% polyacrylamide slab gels, using the Laemmli buffer system.[16] Mol-wt marker proteins ranging from 14,500 to 200,000 daltons were also run on each gel in a separate lane. Each sample was 40 μL (corresponding to 120 μL of the quenched-flow effluent and about 25 μg protein), but in addition, the Coomassie-stained and dried gels were scanned using a densitometer linked to an integrator to determine the relative protein contents of each lane. Later, an autoradiograph peak that correlated directly with protein was identified and used for normalization. The gels were exposed to Kodak XAR-5 film for autoradiography, and the autoradiographs were scanned with the integrator-linked densitometer to assess $[^{32}P]$-labeling of the protein bands.

Cyclic AMP analysis. Supernatants from the trichloroacetic acid-quenched samples were extracted with ether and cAMP determined by automated radioimmunoassay procedures.[17]

Statistical analysis. Student's *t* test for paired samples, when appropriate, was used to examine statistical significances of differences related to thrombin concentration, exposure time, or the presence of external calcium. For simplicity, standard deviations have been excluded from the figures. However, values for one standard deviation are included in the figure legends.

Human subjects. Investigations were performed after approval by the local Human Investigation Committee and in accord with an assurance filed with and approved by the Department of Health, Education, and Welfare.

RESULTS

Earlier experiments[4-6,18] on platelet function suggested that activation of metabolism would begin well within one second of cell stimulation. Consequently, we have used shorter reaction loops[4] to determine whether platelet aggregation, protein phosphorylation, and serotonin secretion might occur as soon as 0.3 second after activation.

Platelet aggregation. Figure 1 shows the effect of thrombin concentration on the time course of aggregation during the first five seconds after stimulation with varying concentrations of thrombin. Control experiments in which platelets were pumped through the apparatus, with saline as the "stimulating agent," reveal no significant shear-induced aggregation. Addition of 0.1 U/mL thrombin did not cause significant aggregation until three seconds, when 20% aggregation was observed. At five seconds, aggregation was reduced to 10%, indicating some disaggregation (data not shown). Higher concentrations of thrombin induced significant aggregation by 0.3 second, and at one second, aggregation induced by 1 or 5 U/mL thrombin was significantly greater than that caused by 0.5 U/mL thrombin. Because differences in aggregation between 1 and 5 U/mL thrombin were insignificant at all time points, the aggregation trace for 1 U/mL thrombin was excluded from the figure.

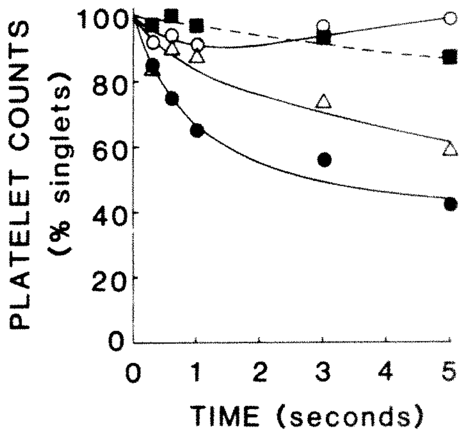

Fig 1. **Influence of thrombin concentration on early platelet aggregation kinetics. $[^{32}P]$-labeled PRE was reacted with varying concentrations of thrombin in the quenched-flow apparatus and quenched with glutaraldehyde (1% final concentration). The effluent at the various reaction times was collected and samples taken for platelet singlet counting to determine the extent of aggregation. In some experiments, the PRE was preincubated for five minutes with 2 mm EDTA to chelate external calcium. The temperature was 37 °C and the data are means from 15 control experiments, eight experiments varying thrombin from 0.1 to 1 U/mL and seven experiments using 5 U/mL thrombin. Standard deviations ranged from 5.5% to 39.2% of the singlet count values, generally increasing with either thrombin or time. The average platelet counts were 332,770/μL (control), 382,307/μL (0.1 to 1 U/mL thrombin), and 284,426 (5 U/mL thrombin). Concentrations of thrombin in the reaction loop were (○), control (saline); (△), 0.5 U/mL; (●), 5 U/mL; and (■, -----), 5 U/mL (PRE preincubated with 2 mmol/L EDTA).**

Serotonin secretion. Kinetics of platelet serotonin secretion were measured using 5 U/mL thrombin as the stimulus (Fig 2). At 0.3 second, 10% of the total $[^{14}C]$-serotonin absorbed by platelets had been secreted ($P < .025$), and by five seconds, 58% of serotonin was lost ($P < .001$). The time course and extent of secretion in our washed platelet preparation (PRE) were similar to that of platelets exposed to thrombin in PRP.[6] Aggregation of the $[^{14}C]$-serotonin–labeled platelets was similar to that of $[^{32}P]$-labeled platelets.

Protein phosphorylation. For thrombin, increases in phosphorylation of up to five proteins have been reported,[19,20] with changes for two proteins of 20,000 and 40,000 to 47,000 mol wt repeatedly noted.[19-27] In our gel system, the 40K to 47K protein had an apparent mol wt of 44,000 daltons, but to save confusion in the discussion, it will be referred to as the 40K protein. We have therefore examined the phosphoryla-

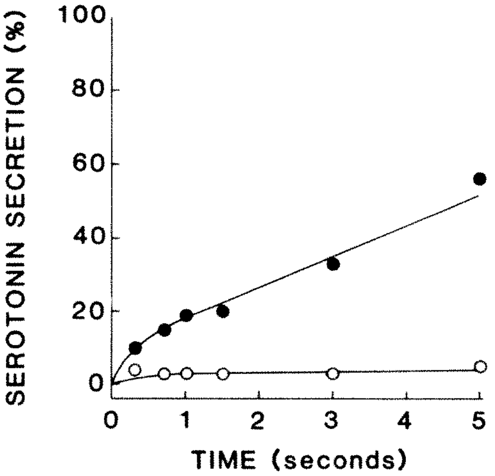

Fig 2. Early kinetics of platelet release of $[^{14}C]$-serotonin in response to thrombin. Washed platelet suspensions, which had been labeled with $[^{14}C]$-serotonin, were reacted with thrombin (5 U/mL) in the quenched-flow apparatus and quenched with 1% glutaraldehyde as described for Fig 1. After samples were taken for platelet counting to determine the extent of aggregation, the remaining material was centrifuged for evaluating $[^{14}C]$-serotonin release, expressed as a percentage of the total platelet content of $[^{14}C]$-serotonin. The temperature was 37 °C and the average platelet count was 364,000/μL. Standard deviations averaged 57.8% of the radioactivity values and showed no dependence on time or thrombin concentration. Values are the means from five preparations of Eagles-PRP: (○), control (saline treated); (●), thrombin treated.

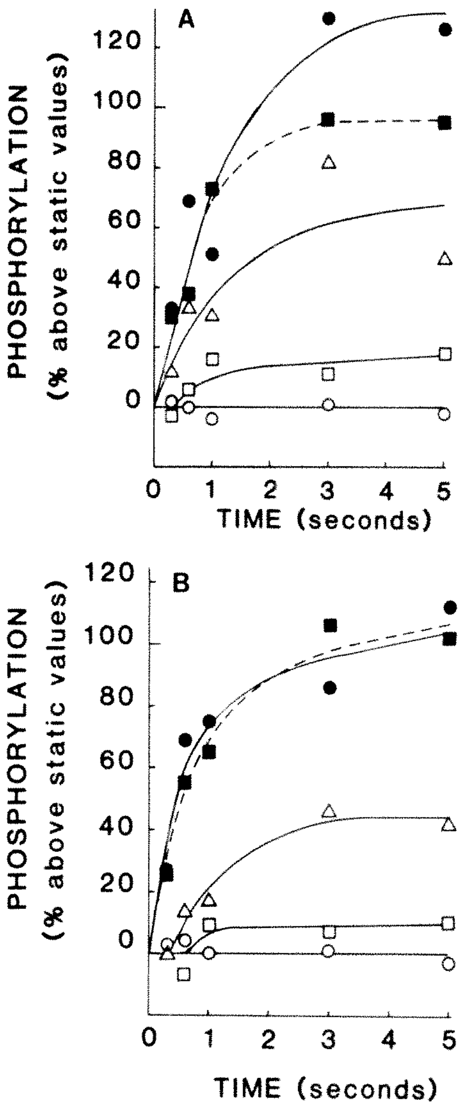

Fig 3. Influence of thrombin concentration on early kinetics of phosphorylation of two platelet proteins: (A) the 20K protein and (B) the 40K protein. $[^{32}P]$-labeled PRE was reacted with varying concentrations of thrombin in the quenched-flow apparatus and quenched with 3N perchloric acid. In some experiments the PRE was preincubated for five minutes with 2mm EDTA to chelate external calcium. The temperature was 37 °C. Protein samples from the quenched effluent were collected and electrophoresed as described in Materials and Methods. The data are derived from autoradiograms of the Coomassie-stained and dried gels that have been scanned and the various peaks integrated and normalized for protein. The means are from 15 control experiments, eight experiments varying thrombin from 0.1 to 1.0 U/mL and seven experiments using 5 U/mL thrombin. The data are expressed as the percentage increase in phosphorylation over the value obtained from platelets not subjected to quenched-flow or exposure to thrombin. Standard deviations ranged from 10.3% to 61.5% of the normalized phosphorylation values, generally increasing with either thrombin or time. Platelet counts were the same as in Fig 1. Concentrations of thrombin in the reaction loop were (○), control (saline); (□), 0.1 U/mL; (△), 0.5 U/mL; (●), 5 U/mL; and (■, -----), 5 U/mL (PRE preincubated with 2 mmol/L EDTA).

tion of these two proteins in the first five seconds after stimulation using varying concentrations of thrombin (0.1 to 5 U/mL). The 5 U/mL concentration caused a maximum response and is a level not very different from what can be formed under in vivo conditions.[15] Phosphorylation induced by 5 U/mL thrombin was compared in the presence and absence of external Ca^{++}, using EDTA.

Figures 3A and B show the progress of these phosphorylations from 0.3 to 5 seconds after activation. With 0.1 U/mL thrombin, increase in phosphorylation of the 20K protein (Fig 3A) became significant ($P < .05$) by 1.0 second and remained constant until five seconds. In contrast, phosphorylation of the 40K protein (Fig 3B) was not significantly greater than the control until five seconds ($P < .05$). The 0.5 U/mL thrombin concentration increased (12%, $P < .05$) phosphorylation of the 20K protein at 0.3 second. By 0.6 second, this was 33% greater than the control value and continued to increase to about 50% above control at five seconds. The higher concentrations of thrombin (1 or 5 U/mL) induced essentially identical increases in 20K phosphorylation. Only the time course for 5 U/mL of thrombin is shown in Fig 3a. Phosphorylation was 33% above control at 0.3 second and continued to increase through three seconds, when it was about 130% greater than control.

Unlike 0.1 and 0.5 U/mL thrombin, higher concentrations increased 40K phosphorylation within 0.3 second (25% ± 34% and 27% ± 27%, respectively, $P < .05$). At 0.6 second, the increase caused by 5 U/mL thrombin was greater than that caused by 1 U/mL, and this difference continued through five seconds. However, the difference was never significant and only the time course for 5 U/mL thrombin is shown. Eighty percent of the total increase in phosphorylation occurred within the first second for these thrombin concentrations.

Dose–response relationship for aggregation and phosphorylation. The concentration of thrombin required to give half-maximal aggregation or phosphorylation response was derived from the dose–response curves for the various time points. The dose–response curves for the 0.6-second time point are shown in Fig 4. The average concentration required to give half-maximal response was 0.51 ± 0.18 U/mL thrombin for aggregation, 0.53 ± 0.16 U/mL for 20K phosphorylation, and 1.10 ± 0.44 U/mL for 40K phosphorylation. The half-maximal concentration for 40K was significantly different from that for 20K phosphorylation or aggregation ($P < .025$).

Significance of external Ca^{++}. Figure 5 shows an autoradiograph and the corresponding Coomassie-stained gel from an experiment to investigate the effect of preincubation with 2 mmol/L EDTA on aggregation and protein phosphorylation. The absence of external Ca^{++} caused by EDTA prevented aggregation by 5 U/mL thrombin during the first three seconds after stimulation (Fig 1, dotted line). By five seconds, a small amount of aggregation occurred, approximately equivalent to that caused by 0.1 U/mL of thrombin. EDTA had no effect on the increase in 40K phosphorylation induced by 5 U/mL thrombin (Fig 3B). The increase in phosphorylation of the 20K protein, however, was significantly reduced ($P < .05$) by an amount approximately 30% of the control value (Fig 3A). This difference was first evident at 0.6 second and did not increase through five seconds.

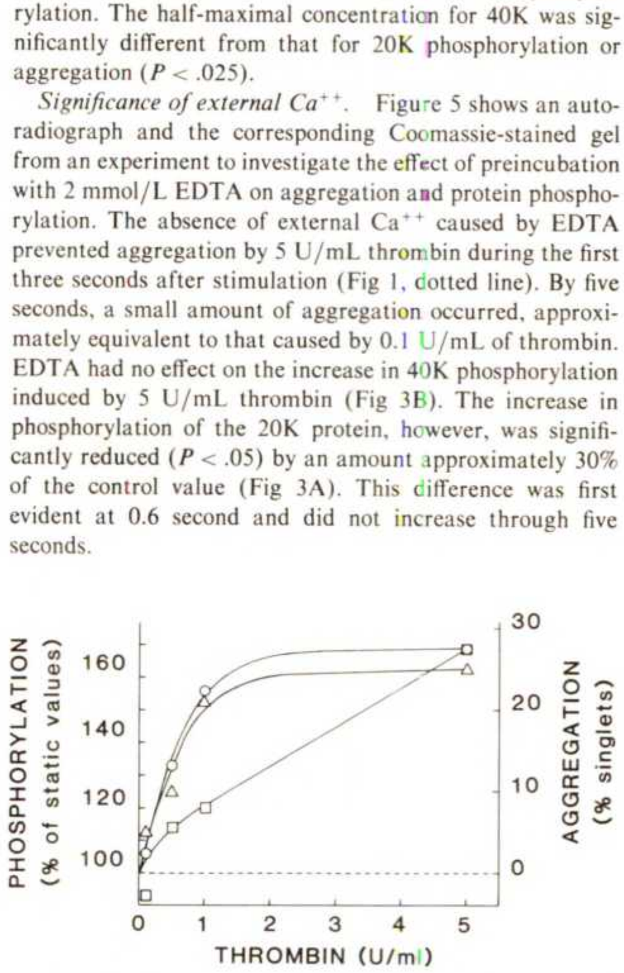

Fig 4. Thrombin dose–response curves for aggregation, 20K phosphorylation, and 40K phosphorylation at 0.6-second exposure to thrombin. The data are means from eight experiments varying thrombin from 0.1 to 1.0 U/mL and seven experiments using 5 U/mL thrombin. Phosphorylation is expressed as a percentage of the value obtained from platelets not subjected to quenched-flow or thrombin, and aggregation is expressed as the percentage reduction in platelet singlets compared with the initial (0 time) count. Standard deviations ranged from 7.0% to 20.1% of the singlet count values and from 12.8% to 61.5% of the normalized phosphorylation values, generally increasing with time. Platelet counts were the same as in Fig 1. The symbols are (○), 20K phosphorylation; (□), 40K phosphorylation; and (△), aggregation.

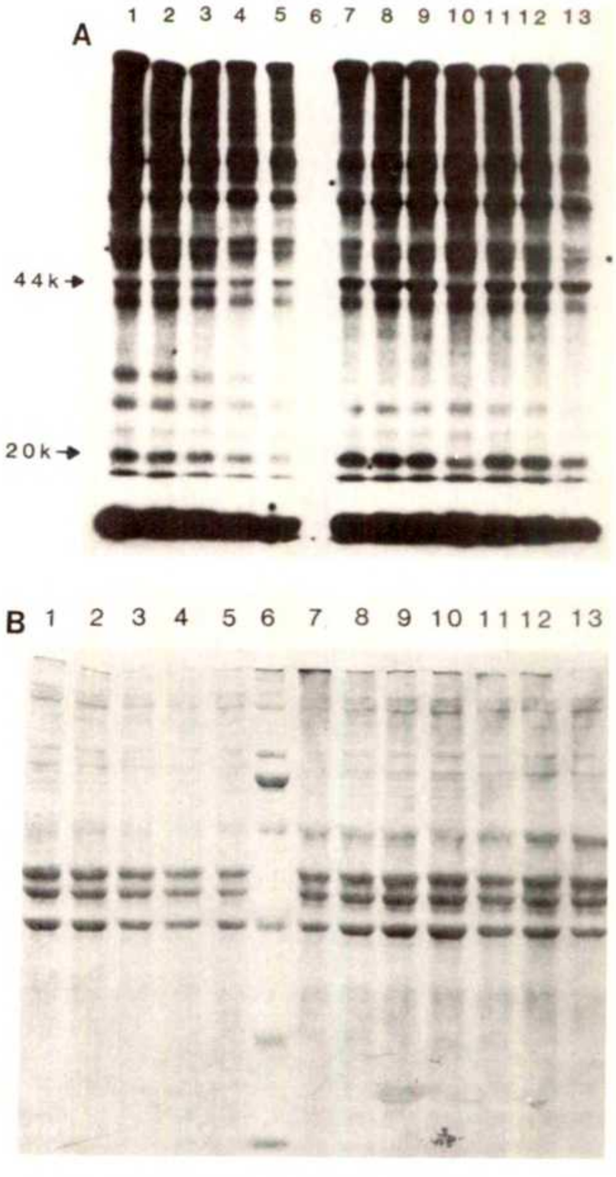

Fig 5. Autoradiogram and slab gel of [^{32}P]-labeled platelets showing the effect of EDTA on thrombin-induced protein phosphorylation. In interpreting the data, autoradiographic densities were always normalized for protein as described in Materials and Methods. (A) Autoradiograms. Lane 1 corresponds to untreated (static) platelets, and lanes 2 through 4, to control platelets pumped through the quenched-flow apparatus with only saline for 1.5, 3, or 5 seconds. Lane 5 corresponds to platelets preincubated with 2 mmol/L EDTA and untreated further. Lane 6 contained standard mol wt marker proteins (unlabeled). Lanes 7 through 9 correspond to platelets preincubated with 2 mmol/L EDTA and subjected to quenched-flow with 5 U/mL thrombin for 1.5, 3, or 5 seconds. Lane 10 corresponds to untreated platelets, and lanes 11 through 13, to platelets exposed to 5 U/mL thrombin for 1.5, 3, or 5 seconds. (B) Coomassie-stained slab gel from which the autoradiogram was derived. Mol-wt markers (lane 6) were 24,000, 34,000, 45,000, 66,200, 92,500, 116,250, and 200,000 daltons.

DISCUSSION

There is evidence that rapid platelet activation is necessary for efficient hemostasis in vivo.[7,8] In fact, activation times as short as two microseconds have been suggested. Yet there have been few reports of biochemical or morphological changes in platelets during the first seconds after stimulation. Mills[18] showed that ADP causes hydrolysis of metabolic adenosine triphosphate (ATP) within a second, and Horne et al[28] have reported changes in cytoplasmic pH and membrane potential as early as five seconds after stimulation with thrombin. Thrombin also increases cytoplasmic calcium levels within two seconds.[29]

As in other cell types, such stimulated Ca^{++} fluxes are accompanied by turnover of phosphatidylinositol. Billah and Lapetina[30] have shown changes in ^{32}P labeling of phosphatidylinositol, phosphatidylinositol 4,5-bisphosphate (PIP_2), and phosphatidic acid as early as five seconds after stimulation, and Kawahara et al[24] have shown a precipitous rise in diglyceride production during the same time period.

Although several researchers have studied the progression of 20K and 40K phosphorylation,[19-25,31-36] activation periods of less than five seconds have seldom have been included. These studies, involving thrombin concentrations from 0.04 to 1 U/mL, indicated that phosphorylation generally approached a maximum at ten to 20 seconds, partially preceding aggregation, measured turbidometrically, and secretion. In some studies, thrombin levels were varied while the exposure period was held constant at a time between 30 seconds and five minutes.[20,21,35,36] The concentration eliciting maximal phosphorylation was reported to vary from 0.1 to 5 U/mL, but no relationship to exposure time was evident.

Our present studies (Fig 3A and B) have indicated that protein phosphorylation can begin within one second of thrombin activation of platelets. It was also noteworthy that the 20K and 40K proteins responded quite distinctly to varying thrombin concentrations. The 20K protein responded to low doses by 0.3 second with little lag, and its K_a of 0.53 U/mL was about one half that for the 40K protein. The latter exhibited a significant lag before slowly responding to low doses of thrombin.

The concentration of thrombin appears to determine not only the final extent of phosphorylation of the 20K and 40K proteins, but also the onset time and initial rate of phosphorylation. A mechanism for shutting down the phosphorylation once begun, but not reversing it, is also indicated. The lower thrombin concentrations caused a steady level of phosphorylation far below the maximum, and the lower the thrombin concentrations, the sooner this stabilization was achieved.

Another distinction in early phosphorylation kinetics for the 20K and 40K proteins was found in the effect of thrombin in the absence of external calcium. EDTA significantly inhibited 20K phosphorylation but had no effect on the 40K protein. External calcium thus appears necessary for maximal phosphorylation of the 20K protein, or myosin light chain. The difference may also be related to the observation that the 20K protein can be partially phosphorylated by the C-kinase enzyme.[32]

The 40K protein is phosphorylated by a phospholipid-dependent, Ca^{++}-activated protein kinase C.[33,34] The functional significance of this is not known, although it appears to be loosely associated with platelet secretion.[19-21,23] It has been suggested that the 40K protein may play a role in the control of phospholipase A_2 (PLA_2) activity,[37] since it is immunoprecipitated by a monoclonal antibody against lipocortin, a known inhibitor of PLA_2. The inhibitory activity of lipocortin is decreased by protein kinase C-induced phosphorylation, and a similar mechanism is suggested for the 40K protein.

Although calcium is involved in both phosphorylations, through different routes, it may be a more important controlling factor for 20K phosphorylation. Phosphorylation of the 40K protein can be modulated by diacylglycerol produced by way of phospholipase C activity,[33,34,38] in such a way that normal cell levels of Ca^{++} are sufficient for full activity. Our data (Fig 3B) indicate that phosphorylation of the 40K protein is independent of external Ca^{++}, at least in the first five seconds after stimulation.

It is interesting to note the similar K_a's for 20K phosphorylation (0.53 ± 0.16 U/mL thrombin) and platelet aggregation (0.51 ± 0.018 U/mL thrombin). The 20K protein has been identified as the myosin light chain, and its early phosphorylation is catalyzed by the calmodulin-dependent myosin light-chain kinase. This phosphorylation is necessary for the ATPase activity and force generation of platelet actomyosin,[39] and its occurrence is associated with incorporation of myosin light chains into the platelet cytoskeleton.[31] Myosin light-chain dephosphorylation is associated with platelet aggregation.[26] However, this dephosphorylation occurred at three to five minutes after stimulation, well outside the range of our observations.

These studies provide evidence for early biochemical events in the stimulus–response sequence in blood platelets. The time scale approaches that needed for efficient hemostasis in vivo[7,8] and complements our earlier studies[4-6] of shape change, aggregation, and secretion in PRP. Because mixing may be more efficient in our system, activation may occur earlier than with other methods, but it is the earliest biochemical events, those occurring before or during shape change, aggregation, and secretion, that can be expected to be most relevant to platelet function. It will be instructive to correlate cytoskeleton dynamics, Ca^{++} movements, phospholipid turnover, and the activities of phospholipases with this new information.

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REFERENCES

1. Berridge MJ: Receptors and calcium signaling. Trends Pharmacol Sci 1:419, 1980

2. Michell RH: Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta 415:81, 1975

3. Bithell TC: The physiology of primary hemostasis, in Wintrobe MM, Lee GR, Boggs DR, Bithel TC, Foerester J, Athens JW, Lukens JN (eds): Clinical Hematology (ed 8). Philadelphia, Lea & Febiger, 1981, p 390

4. Gear ARL: Rapid reactions of platelets studied by a quenched-flow approach: Aggregation kinetics. J Lab Clin Med 100:866, 1982

5. Gear ARL: Rapid platelet morphological changes visualized by scanning electron microscopy: Kinetics derived from a quenched-flow approach. Br J Haematol 56:387, 1984

6. Gear ARL, Burke DJ: Thrombin-induced secretion of serotonin from platelets can occur in seconds. Blood 60:1231, 1982

7. Born GVR: Fluid-mechanical and biochemical interactions in haemostasis. Br Med Bull 33:193, 1977

8. Born GVR, Gorog P, Kratyer MAA: Aggregation of platelets in damaged vessels. Philos Trans R Soc Lond [Biol] B 294:241, 1981

9. Carty DJ, Spielberg F, Gear ARL: Thrombin causes subsecond changes in protein phosphorylation of platelets. Fed Proc 43:977, 1984

10. Carty DJ, Gear ARL: Rapid changes in platelet protein phosphorylation induced by thrombin, ADP and epinephrine, as studied by quenched-flow techniques. Thromb Haemost 54:14, 1985

11. Haver VM, Gear ARL: Functional fractionation of platelets. J Lab Clin Med 97:187, 1981

12. Haver VM, Gear ARL: Functional fractionation of platelets: Aggregation kinetics and glycoprotein labeling of differing platelet populations. Thromb Haemost 48:211, 1982

13. Han P, Ardlie N: The influence of pH, temperature and calcium on platelet aggregation. Maintenance of environmental pH and platelet function for *in-vitro* studies in plasma stored at 37 °C. Br J Haematol 26:373, 1974

14. Packham MA, Guccione MA, Greenberg JP, Kinlough-Rathbone RL, Mustard JF: Release of ^{14}C-serotonin during initial platelet changes induced by thrombin, collagen, or A23187. Blood 50:915, 1977

15. Fenton JW: Thrombin specificity. Ann NY Acad Sci 370:468, 1981

16. Laemmli U: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680, 1970

17. Brooker G, Teresaki W, Price M: Gammaflow: A completely automated radioimmunoassay system. Science 194:270, 1976

18. Mills DCB: Changes in the adenylate energy charge in human blood platelets induced by adenosine diphosphate. Nature 243:220, 1973

19. Haslam RJ, Lynham JA: Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents. I. Effect of different aggregating agents. Biochem Biophys Res Commun 77:714, 1977

20. Wallace WC, Bensusan HB: Protein phosphorylation in platelets stimulated by immobilized thrombin at 37 ° and 4 °C. J Biol Chem 255:1932, 1980

21. Wallace WC, Bensusan HB: The effects of platelet secretion inhibitors on protein phosphorylation. Biochim Biophys Acta 721:164, 1982

22. Lyons L, Stanford N, Majerus PW: Thrombin-induced protein phosphorylation in human platelets. J Clin Invest 56:924, 1975

23. Bennett WF, Belville JS, Lynch G: A study of protein phosphorylation in shape change and Ca^{++} dependent serotonin release by blood platelets. Cell 18:1015, 1979

24. Kawahara Y, Takai Y, Minakuchi R, Sano K, Yasutomi N: Phospholipid turnover as a possible transmembrane signal for protein phosphorylation during human platelet activation by thrombin. Biochem Biophys Res Commun 97:309, 1980

25. Daniel JL, Molish IR, Holmsen H: Myosin phosphorylation in intact platelets. J Biol Chem 256:7510, 1981

26. Carroll RC, Gerrard JM: Phosphorylation of platelet actin-binding protein during platelet activation. Blood 59:466, 1982

27. Feinstein MB, Egan JJ, Opas EE: Reversal of thrombin-induced myosin phosphorylation and the assembly of cytoskeletal structures in platelets by adenylate cyclase stimulants prostaglandin D_2 and forskolin. J Biol Chem 58:1260, 1983

28. Horne WC, Norman NE, Schwartz DB, Simons ER: Changes in cytoplasmic pH and membrane potential in thrombin-stimulated human platelets. Eur J Biochem 120:295, 1981

29. Feinstein MB, Walenga R: The role of calcium in platelet activation. KROC Found Ser 14:279, 1981

30. Billah MM, Lapetina EG: Rapid decrease of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. J Biol Chem 257:12705, 1982

31. Fox JEB, Phillips DR: Role of phosphorylation in mediating the association of myosin with the cytoskeletal structures of human platelets. J Biol Chem 257:4120, 1982

32. Naka M, Nishikawa M, Adelstein RS, Hidaka H: Phorbol ester-induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. Nature 306:490, 1983

33. Kaibuchi K, Takai Y, Sawamura M, Hoshijima M, Fujikura T, Nishizuka Y: Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J Biol Chem 258:6701, 1983

34. Ieyasu H, Takai Y, Kaibuchi I, Sawamura M, Nishizuka Y: A role of calcium-activated, phospholipid-dependent protein kinase in platelet-activating factor-induced serotonin release from rabbit platelets. Biochem Biophys Res Commun 108:1701, 1982

35. Sano K, Takai Y, Yamanishi J, Nishizuka Y: A role of calcium-activated phospholipid-dependent protein kinase in human platelet activation. Comparison of thrombin and collagen actions. J Biol Chem 258:2010, 1983

36. Kawahara K: A possible role of calcium-activated, phospholipid-dependent protein kinase in platelet activation. Kobe J Med Sci 27:119, 1982

37. Touqui L, Rothhut B, Shaw A, Fradlin A, Vargaftig BB, Russo-Marie F: Presence of antiphospholipase proteins (lipocortin) in platelets: Its regulation by protein kinase C-dependent phosphorylation. Thromb Haemost 54:220, 1985

38. Takai Y, Kishimoto A, Kikkawa V, Mori T, Nishizuka Y: Unsaturated diacylglycerol as a possible messenger for the activation of calcium-activated, phospholipid-dependent protein kinase C. Biochem Biophys Res Commun 91:1218, 1979

39. Gerrard JM, Carroll RC: Stimulation of platelet protein phosphorylation by arachidonic acid and endoperoxide analogues. Prostaglandins 22:81, 1981

Substrate for Endothelial Prostacyclin Production in the Presence of Platelets Exposed to Collagen Is Derived From the Platelets Rather Than the Endothelium

By C.N. Chesterman, R. Owe-Young, Janet Macpherson, and S.A. Krilis

Interactions between vascular endothelial cells and blood platelets have been investigated using a model microcirculation consisting of microcarrier beads colonized with human umbilical vein endothelial cells (HUVECs) and perfused with washed platelet suspensions. To simulate the effects of endothelial desquamation and exposure of subendothelium, fibrillar collagen in suspension was coinjected with the platelets. In this model, neither the passage of platelets alone nor collagen alone stimulated prostacyclin (PGI_2) production by the HUVECs. Platelets activated by coinjection with collagen released thromboxane A_2 (TXA_2), and this was associated with the simultaneous production of PGI_2 by the HUVECs. By means of double-isotope experiments with [^3H]arachidonic acid (AA) incorporated into platelets and [^{14}C]-AA into HUVECs, it was shown that all the PGI_2 generated was derived from platelet AA and/or endoperoxides. This interpretation was strengthened by the finding that PGI_2 production was not prevented by treatment of HUVECs with indomethacin followed by perfusion with collagen-stimulated platelets. AA metabolites in double-isotope label experiments were further characterized by reverse-phase chromatography, and it was shown that both cyclooxygenase and lipoxygenase products of the HUVECs were derived from platelet membrane lipid. Thrombin regularly produced transient PGI_2 release, but showed rapid tachyphylaxis. Platelet-derived compounds including ADP, ATP, and platelet-activating factor (PAF) did not produce PGI_2 release by HUVECs in this system. Thus, the transfer of AA and metabolites from collagen-stimulated platelets is likely to be the mechanism for PGI_2 production in the context of minor degrees of endothelial desquamation.

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THE RELATIONSHIP between circulating blood platelets and the vessel wall is complex, but it is clear that endothelial cells maintain a nonadhesive luminal surface, this property relying on a number of unstimulated membrane and metabolic characteristics. The concept that endothelial-derived prostacyclin (PGI_2), continuously released, may result in circulating "anticoagulant" activity[1] has not proved tenable.[2] Measurable levels are not high enough. Nevertheless, PGI_2 release by endothelium is still regarded as a local and effective response to vascular "injury" or thrombotic stimulus, providing a defense against further local platelet deposition and initiating vasodilatation, which might in itself promote blood flow rather than stasis.[2,3] Defining the mechanisms and the agonists responsible for such a local PGI_2 release in a given situation is not as easy as might be expected, because potential stimuli differ and the vascular response to individual agonists varies with both the species and the anatomical origin of the endothelium.[4-6]

If the subendothelium becomes exposed to circulating blood or the endothelium loses its platelet-repelling properties, it is likely in many instances that the only stimulus to PGI_2 production by nearby endothelium is platelet derived. A number of platelet release products have been reported to stimulate endothelial PGI_2 release, including adenine nucleotides, ADP and ATP,[7] platelet-derived growth factor (PDGF),[8] platelet-activating factor (PAF)[6,9] and serotonin,[6] although responses are both species and site dependent. In other situations, further stimulus to PGI_2 production by endothelium may be contributed by other cells: for example, ADP from damaged red cells or PAF and leukotrienes released by granulocytes.

Experiments incubating cultured endothelial cells (EC) with human platelets[10,11] or slices of vascular tissue with platelets[12] have suggested the occurrence of the transfer of cyclic endoperoxides from stimulated platelets to EC, thus providing substrate for PGI_2 production. Some authors, however, have reported results showing that this is not a significant phenomenon.[13-15] For the most part, these experiments were carried out in static systems which suffered from an unfavorable ratio of culture medium to the surface area of the EC and which introduced potential artifacts by the physical or enzymatic manipulation of the cultured cells and from the accumulation of metabolites during the time course of the experiment.

To avoid these problems and to construct a physiological model to investigate interactions between EC and platelets in flowing blood, we used a perfusion system in which the luminal surface of the vessel wall was represented by microcarrier beads colonized with human umbilical vein endothelial cells (HUVECs).[5,16] In this way, we were able to document clearly that the transfer of arachidonate and metabolites from collagen-stimulated platelets to vascular EC is the major mechanism for the production of PGI_2 by EC in the immediate vicinity.

MATERIALS AND METHODS

HUVECs. Endothelial cells were harvested from umbilical cords <4 hours after delivery by collagenase digestion.[17] Primary cultures were initiated in 25-cm^2 culture flasks (Corning, NY). Two culture techniques were used. In the first method, the flasks were incubated overnight with human fibronectin 10 μg per square centimeter. The fibronectin was prepared according to the method of Engvall and Ruoslahti.[18] The culture medium was M199 (Flow Laboratories, North Ryde, Sydney), 0.1% penicillin/streptomycin (GIBCO, Grand Island, NY) 20% human umbilical cord blood serum (T.H. Forster, personal communication), and 5% CO_2 in air at 37 °C.

In the second method, the flasks were incubated overnight with

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1% gelatin (Haemaccel; Behringwerk AG, Marburg, FRG); the medium was M199, 0.1% penicillin/streptomycin, 20% fetal calf serum (FCS) from Flow Laboratories, 20 μg per milliliter endothelial cell growth factor (ECGF) obtained from BRL Laboratories (Gaithersburg, Maryland), heparin 90 μg per milliliter (Weddell, Wrexham, England) in 5% CO_2 in air at 37 °C.[19]

The cells were passaged with trypsin-EDTA (Flow Laboratories) and split in a ratio of 1:3. At passage 3, the contents of 3 × 75 cm^2 flasks of cells were seeded onto 3 g of microcarrier beads (Biosilon, Nunc, Denmark).[5,16] The microcarriers had been preincubated with the respective adhesive protein, and the culture was continued in microcarrier culture bottles (Techne, Cambridge, England), being stirred at 40 rpm for 20 seconds every 30 minutes. The cells on beads were visualized by staining a small aliquot in 0.5% methyl violet for 15 to 20 minutes, rinsing with tap water, and observing under a ×10 objective. HUVEC counts were performed by incubating a small aliquot of colonized beads in trypsin-EDTA at 37 °C for five minutes before the cells were resuspended and counted in a hemocytometer. Factor VIII antigen immunofluorescence was observed using rabbit anti-human factor VIII antigen (Dako, Denmark) and FITC-conjugated sheep anti-rabbit IgG (Miles Laboratories, Mulgrave, Australia).

[^{14}C]-Labeled AA Incorporation into HUVECs. Two and a half microcuries [^{14}C]AA (NEC-756, 390 mCi per millimole Lot No. 1931-109, New England Nuclear, Boston) was evaporated in a Speed Vac concentrator (Savant Instruments Inc, Farmingdale, NY) and then resuspended in 1 mL of serum-free M199. The medium containing the labeled AA was added to 19 mL of serum-free M199 containing 2.65 g of microcarrier beads with ~2.7 × 10^6 HUVECs. The beads bearing the HUVECs had previously been washed twice with phosphate-buffered saline (PBS). The cells were incubated at 37 °C for 75 minutes with intermittent stirring in the culture vessel. Aliquots of beads and medium were taken prior to and after incubation to ascertain cell association of [^{14}C] into the HUVECs. Under these conditions, cell association was ~37%.

Washed platelet suspension. Platelets were prepared by a modification of the method of Vargas and colleagues.[20] Prostacyclin (PGI_2) (Wellcome Laboratories, Kent, England) 300 ng per milliliter was added to citrated platelet-rich-plasma (PRP) and in each of two subsequent washes in Tyrode's buffer, which was supplemented with 0.01% fatty acid-free bovine serum albumin (BSA). Heparin, 25 U per milliliter was included in the first wash. In experiments in which platelets were prelabeled with tritiated AA, the platelets at the second wash were incubated with 50 μCi of tritiated AA (NEN, 83.8 Ci per millimole, NET 298 Lot No. 2145-160) for 45 minutes at 37 °C with intermittent agitation and then pelleted. The platelets were resuspended at 2 × 10^8 per milliliter in PGI_2-free Tyrode's buffer and stored in a double-walled siliconized container at 4 °C overnight before use. Prior to the experiment, platelets were resuspended with gentle pipetting and allowed to come to room temperature. Platelet aggregation was assessed using collagen at 5 or 10 μg/400 μL of platelet suspension.

Platelet aggregation. Platelet aggregation was carried out at 37 °C in a Payton single-channel aggregometer, Model 401 (Payton Associates, Scarborough, Ontario, Canada) with an Omniscribe flatbed recorder, Model EB5117-1 (Houston Inst., Austin, Texas).

Agonists. Agonists used to stimulate either platelet or HUVEC arachidonate metabolism were as follows: Thrombin was of bovine origin (Thrombostat, Parke-Davis, Sydney, Australia); adenosine 5'-diphosphate (ADP) disodium salt was from Calbiochem-Behring (Lot 102030); adenosine 5'-triphosphate (ATP) disodium salt was from Calbiochem-Behring (Lot 102197); PAF (L-α-phosphatidylcholine, β-acetyl-γ-O-alkyl) was from Sigma (St Louis), P9525 (Lot 53F8370) evaporated under N_2 and diluted in tissue culture medium (TCM) containing BSA 2.5 mg per milliliter; collagen suspension was equine in origin in isotonic glucose solution (Hormon-Chemie, Munich, FRG).

Platelet and white cell counts. Cell counts were performed in a Coulter-S plus Particle Counter using Isoton II as diluent (Coulter Electronics, Sydney, Australia).

Thromboxane B_2 (TXB_2) and 6-keto prostaglandin $gF_{1\alpha}$ (6-keto $PGF_{1\alpha}$). Arachidonate metabolites were assayed in unextracted perfusion fractions by radioimmunoassay (RIA) using Seragen antisera (Flow Laboratories). Cross-reactivity with 6-keto-$PGF_{1\alpha}$ antiserum at 50% B/B_0 was stated by the suppliers to be 2.2% for $PGF_{2\alpha}$, 0.6% for PGE_2, and <0.01% for both PGD_2 and TXB_2. Cross-reactivity with the TXB_2 antiserum was stated to be <0.1% for prostaglandins including 6-keto-$PGF_{1\alpha}$ and 5-, 12-, and 15-HETES. When [^{3}H] metabolites were present in column fractions, RIA was performed using [^{125}I]-labeled tracers (New England Nuclear RIA kits). Standard curves were constructed using as diluting buffer the serum-free culture medium and, where appropriate, including a dilution of the anticoagulant mixture used to collect the platelet suspensions from perfusion (see below). When platelets were perfused through the HUVEC column, the eluate 6-keto-$PGF_{1\alpha}$ concentrations were corrected for the small residual contamination in the platelet suspension (see Washed platelet suspension section above).

^{125}I-Assays were counted in an LKB Wallac Gamma Sample Counter Model GTL 300-500 equipped with AS-12 Pulse Height analyser and SC-20/SC-23 scalers.

Analysis of radiolabeled AA metabolites from column fractions. Perfusion fractions were prepared for analysis by extraction in 80% ethanol (vol/vol)[21] and stored at −70 °C under argon before centrifugation at 3,000 g for five minutes, evaporation in the Speed Vac concentrator at 37 °C and reconstituted in 250 μl of starting solvent. The sample was subjected to reverse-phase chromatography on a Pharmacia PepRPC HR 5/5 column, C_2/C_{18}, operating with a Pharmacia FPLC (fast protein liquid chromatography) System. The column was developed with a stepwise methanol gradient as follows: flow rate 0.5 mL/min; methanol 47%, 0 to 30 minutes; methanol 60%, 31 to 65 minutes; methanol 68%, 66 to 85 minutes; methanol 100%, 86 to 95 minutes. The remainder of the solvent was buffered water, acetic acid (1:0.0008) brought to pH 6.2 with a 50% vol/vol solution of 33% ammonia [modified from Henke et al[21]]. Fractions were mixed with 8 vol of Amersham ACSII scintillant and counted in a Packard Minaxi, Model D4430 scintillation counter using a dual-label program in which [^{3}H] and [^{14}C] labeled metabolites were present. Tritiated standards, AA, prostaglandins ($PGF_{2\alpha}$, PGD_2, PGE_2), 5-,12-,15-HETE, and leukotrienes (LTB_4, LTC_4, LTD_4, and LTE_4) were obtained from NEN. 6-Keto $PGF_{1\alpha}$ and TXB_2 were from Amersham (Sydney, Australia). Standard mixtures were subjected to extraction procedures identical to perfusion fractions. High-performance liquid chromatography (HPLC) grade methanol was obtained from Mallinckrodt (South Oakleigh, Victoria), HPLC grade acetic acid from Ajax Chemicals (Sydney), and ethanol (AR grade) from May & Baker (Melbourne). Water was triple-distilled, deionized using Elgacan C114 cartridge, and filtered through a Millipore 0.22-μm filter.

The recoveries of total DPM of radiolabeled standards 6-keto-$PGF_{1\alpha}$, TXB_2, 12-HETE, and 15-HETE following extraction from M199 and reverse-phase chromatography were 87.9%, 93.3%, 98%, and 96%, respectively. Recoveries of the standards from Tyrode's buffer, 0.01% BSA, were 88.8%, 90.2%, 99.2% and 93.2%, respectively.

Experimental perfusion system. The system is illustrated diagramatically in Fig 1. A perspex column with an internal diameter of 4 mm and a length of 90 mm accommodated ~1 g of microcarrier beads colonized with HUVECs or, in control situations, beads alone coated with fibronectin or gelatin. The column could be extended

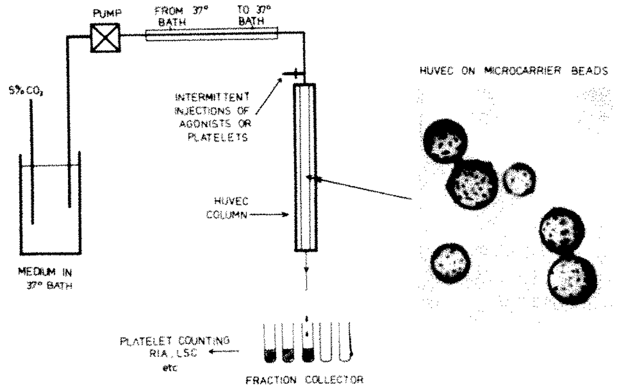
PUMP
FROM 37° BATH
TO 37° BATH
5% CO₂
INTERMITTENT INJECTIONS OF AGONISTS OR PLATELETS
HUVEC COLUMN
MEDIUM IN 37° BATH
HUVEC ON MICROCARRIER BEADS
PLATELET COUNTING RIA, LSC etc
FRACTION COLLECTOR

Fig 1. Diagram of the system used to perfuse human umbilical vein endothelial cells colonized on microcarrier beads packed in a perspex column. Two columns of beads were perfused simultaneously.

and doubled in length if required. A Holter pump (model 912) was used to perfuse two columns simultaneously with serum-free M199 at 0.4 to 0.6 ml/min. The medium was gassed with 5% CO_2 in a 37 °C water bath and was delivered to the column at 37 °C by passage through a heated water jacket. An injection of prewarmed agonists or platelet suspensions was made into a side arm at the top of the column using a 1-mL tuberculin syringe, the flow rate being maintained. Column perfusate was collected in 1-mL fractions. In experiments in which nucleotides were used as agonists, columns were perfused with Dulbecco's modified Eagle's medium (DMEM) because this medium contains no added nucleotides. If a platelet suspension was perfused through the column, an aliquot of each fraction was taken into Isoton for platelet counting. To the remainder of the fraction was added EDTA/theophylline/prostaglandin E_1 (ETP)[22] anticoagulant brought to 4 °C and centrifuged at 11,000 g for five minutes before the supernatant was taken off for assay. The perfusion system was cleaned with 0.1 mol/L of HCl between experiments, and sterility was maintained during perfusion by aseptic technique.

RESULTS

HUVECs were regularly used at the third passage to provide adequate numbers of cells on microcarriers. The mean number of cells per column was $3.07 \pm 1.58 \times 10^6$ (N = 17). Both culture methods resulted in similar proliferation rates, 5.3 ± 2.8 days (N = 22) between passages in media supplemented with CBS and 4.6 ± 2.1 (N = 28) days with fetal calf serum/endothelial cell growth factor (FCS/ECGF) heparin. The morphology of the HUVECs, however, was noticeably different. The cells in CBS maintained typical cobblestone appearance, whereas those grown in FCS/ECGF/heparin became elongated. In both, however, there was expected factor VIII antigen staining by immunofluorescence.

Platelet preparations. The method for platelet preparation was suitable for these studies because the platelets remained as a single cell suspension and retained their reactivity to collagen (and other agonists, although these were not regularly tested). The platelet recovery was 58.2% ± 8.6% (N = 16) of the initial PRP, and there was contamination with 3 ± 1.3 white cells per 10^3 platelets (N = 9). The uptake of [^{3}H] AA, when included, was between 50% and 60%.

Experiments with unlabeled platelet suspensions passed through the HUVEC column. A 1-mL suspension of 2×10^8 platelets was fully recovered after passage through the HUVEC column over six fractions (6 mL) (Fig 2A). There was the concurrent release of 0.63 ng TXB_2. When 10 μg of collagen suspension was coinjected with 2×10^8 platelets (from the same platelet preparation), 55% of the platelets were retained in the column. This was accompanied by 7.71 ng TXB_2 and a simultaneous PGI_2 release measured as 3.7 ng 6-keto-$PGF_{1\alpha}$. A control column perfused with collagen (10 μg) suspension produced no 6-keto-$PGF_{1\alpha}$.

When 6×10^8 platelets coinjected with 30 μg collagen suspension were passed over a HUVEC column that had been pretreated with 10 μmol/L of indomethacin for 60 minutes, there was 6-keto-$PGF_{1\alpha}$ production (12.3 ng) and retention of 30% of the platelets (Fig 2B). In experiments in which platelet suspensions were coinjected with collagen (examples Figs 2 and 3), although there was a net retention of platelets during the perfusion, there was a tendency for platelets to continue to appear from the column over a period of 10 to 20 minutes in some instances (Fig 3) with a second peak following the release of PGI_2, suggesting the possibility of disaggregation or the release of adherent platelets.

Isotopically [^{3}H]-AA-labeled platelets and [^{14}C]-AA-labeled HUVECs interaction. A 10-minute perfusion of a HUVEC column was carried out with a total of 10^9 platelets (2×10^8/mL) coinjected with 50 μg of collagen suspension. The platelets had been incubated with [^{3}H]-AA for 45

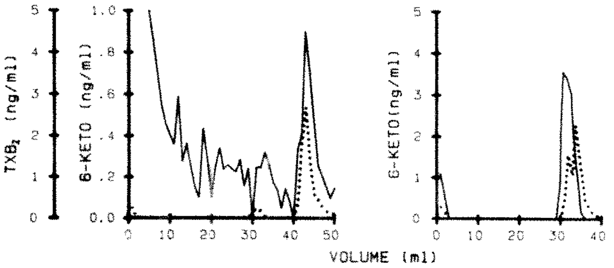

Fig 2. (A) One milliliter of Tyrode's buffer containing 2×10^8 platelets in suspension was perfused through a column containing human umbilical vein endothelial cells on microcarrier beads over 2 minutes, the column otherwise being continuously perfused with medium 199 at the same flow rate. A second perfusion of platelets, this time with coinjection of 10 μg collagen, followed 20 minutes later. The platelet recovery from the column is shown in the upper panel (------). Lower panel: production of thromboxane [radioimmunoassay (RIA) of thromboxane B_2] ...... and prostacyclin (RIA of 6-keto-$PGF_{1\alpha}$) during the experiment. (B) A similar column of human umbilical vein endothelial cells was pretreated with 10 μmol/L of indomethacin for 60 minutes before perfusion with 6×10^8 platelets and 30 μg of collagen over 6 minutes. The symbols are as in A.

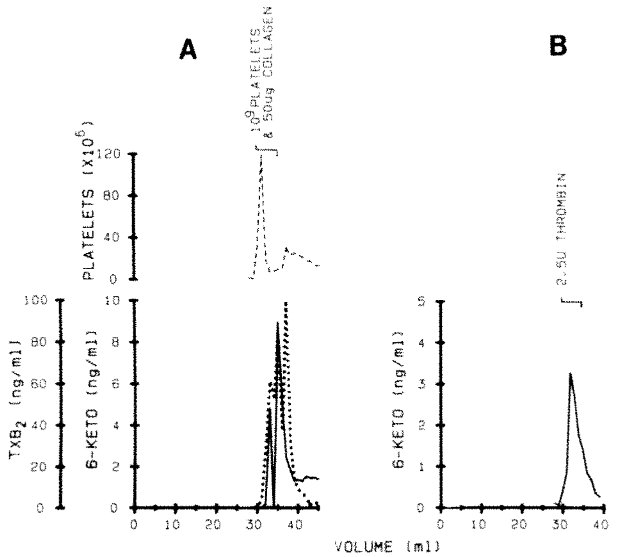

Fig 3. **(A) A 10-minute perfusion of a human umbilical cord endothelial cells (HUVECs) column with a total of 10^9 platelets (coinjected with 50 μg collagen) was carried out. The platelets were prelabeled with [^3H]-arachidonic acid (AA), and the HUVECs were prelabeled with [^{14}C]-AA. The platelet recovery is shown in the upper panel and the production of thromboxane [radioimmunoassay (RIA) of thromboxane B_2] and prostacyclin (RIA of 6-keto-$PGF_{1\alpha}$) shown in lower panel. Symbols as in Fig 2. (B) A similar column of [^{14}C]-AA prelabeled HUVECs was perfused with thrombin, 2.5 U. Results of RIA of 6-keto-$PGF_{1\alpha}$ performed on column fractions are shown.**

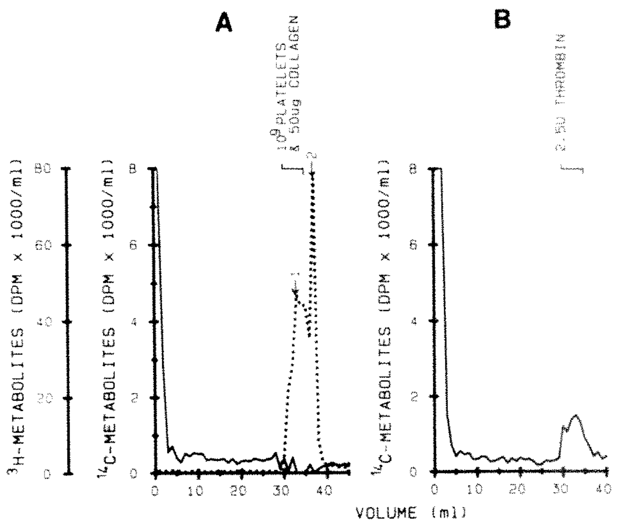

Fig 4. **Aliquots of the human umbilical vein endothelial cell (HUVEC) column fractions from the experiments shown in Fig 3 were counted for the release of [^3H] [platelet-derived arachidonic acid (AA) or metabolites] and for [^{14}C] (HUVEC-derived AA or metabolites) as a response to injections of collagen and platelets (A) or thrombin (B). Peaks labeled 1 and 2 were subjected to reverse phase chromatography (Fig 5).**

minutes and the HUVECs with [^{14}C]-AA for 75 minutes prior to the experiment. Sixty percent of the platelets were retained in the column, and there was simultaneous TXA_2 (518.9 ng) and PGI_2 (33.2 ng) production; the latter persisted for more than 15 minutes after the injection of platelets had finished (Fig 3A). As a control for the ability of the HUVECs to respond, an identical column was perfused with 0.5 U/mL of thrombin for ten minutes (Fig 3B). There was a transient release (12.6 ng) of PGI_2 that was not sustained throughout the injection.

Following centrifugation (as described in Methods section) of the fractions collected during these experiments, aliquots were counted for the release of [^3H]-AA metabolites (platelet-derived) and [^{14}C]-AA metabolites (HUVEC-derived) (Fig 4). The passage of collagen-stimulated platelets resulted in only [^3H]-labeled metabolites (Fig 4A), whereas the injection of thrombin resulted in the release of [^{14}C]-labeled metabolites (Fig 4B).

When an early fraction from the passage of collagen-stimulated platelets (fractions 32 and 33 pooled, marked 1, Fig 4A) was extracted and subjected to reverse-phase (RP) chromatography, the profile demonstrated the following features (Fig 5A): 39.3% of the [^3H]-AA was converted to metabolites. Of the total, 7.4% eluted in the buffer front, 2.9% eluted with a retention time identical to [^3H]-TXB_2, a small peak [unidentified, but presumably 12-hydroxyheptadecatrienoic acid (HHT)] comprised of 3.2% of DPM and the major peak of 10.3% eluted with a retention time similar to that of standard 12-HETE. An identical profile was obtained from similarly prelabeled platelets stimulated by collagen in a column with fibronectin-coated beads without HUVECs.

A later fraction (fraction 37, marked 2, Fig 4A) from the passage of [^3H]-AA platelets (collagen stimulated) and [^{14}C]-AA HUVECs was subjected to RP chromatography. In addition to the TXB_2, probable HHT, and 12-HETE, three new [^3H] peaks were identified (Fig 5B). These corresponded to 6-keto $PGF_{1\alpha}$ (4.4%), PGD_2/PGE_2 (2.6%), and 15-HETE (2.9%), respectively.

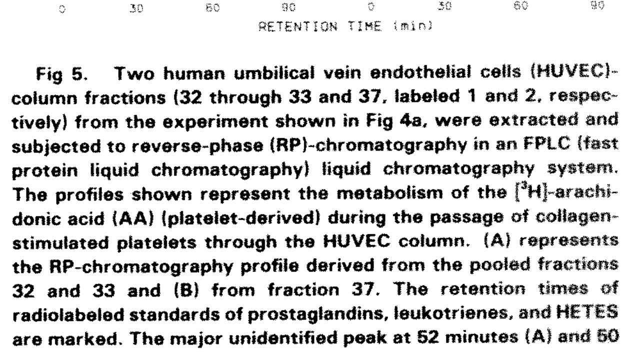

Fig 5. **Two human umbilical vein endothelial cells (HUVEC)-column fractions (32 through 33 and 37, labeled 1 and 2, respectively) from the experiment shown in Fig 4a, were extracted and subjected to reverse-phase (RP)-chromatography in an FPLC (fast protein liquid chromatography) liquid chromatography system. The profiles shown represent the metabolism of the [^3H]-arachidonic acid (AA) (platelet-derived) during the passage of collagen-stimulated platelets through the HUVEC column. (A) represents the RP-chromatography profile derived from the pooled fractions 32 and 33 and (B) from fraction 37. The retention times of radiolabeled standards of prostaglandins, leukotrienes, and HETES are marked. The major unidentified peak at 52 minutes (A) and 50 minutes (B) is probably 12-hydroxyheptadecatrienoic acid.**

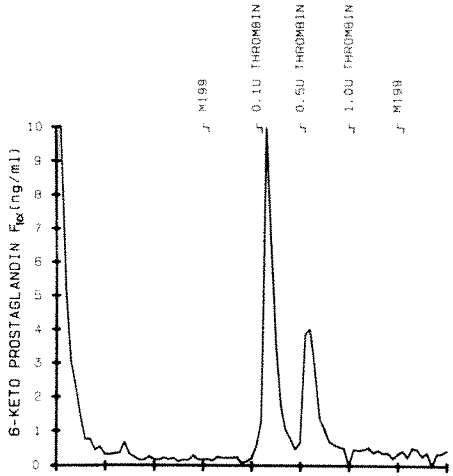

Fig 6. A column of human umbilical vein endothelial cells was stimulated with three successive doses of thrombin (0.1 U, 0.5 U, 1.0 U) at 20-minute intervals. The resulting prostacyclin production (radioimmunoassay of 6-keto-$PGF_{1\alpha}$) is shown.

Prostacyclin released by HUVECs to perfusion with agonists. Following institution of flow through the perfusion system, a substantial but short-lived release of PGI_2 as measured by 6-keto $PGF_{1\alpha}$ was invariable [20.2 ± 6.4 ng, (N = 6)]. After 20-minute perfusion, little or no 6-keto $PGF_{1\alpha}$ was detectable in the perfusate. On 10 of 18 occasions, the minimal change in the flow due to the control injection of culture medium alone resulted in a transient release of PGI_2 [1.6 ± 2.5 ng (N = 18)]. Thrombin, 0.1 to 0.5 U always resulted in substantial but transient release of PGI_2 [5.73 ± 7.4 ng (N = 14)]. A dose-response using the same column of cells was not constructed because there was rapid desensitization of the HUVECs so that a second injection of thrombin released little or no PGI_2 (Fig 6). Other putative agonists assessed, ADP (10^{-3} to 10^{-6} mol/L), ATP (10^{-3} to 10^{-6} mol/L) and PAF (10^{-5} to 10^{-8} mol/L) produced occasional but no reproducible responses in excess of control injections (N = 44).

DISCUSSION

We have shown that intact, washed platelets pass through a HUVEC column without significant activation and without adherence to components in the column. There was no release of PGI_2 by the EC as a consequence of the proximity of unactivated platelets, nor did fibrillar collagen in suspension result in PGI_2 release by the ECs. When the collagen, representing local subendothelium in this model, was coinjected with platelet suspension, there were physical and metabolic consequences. About 50% of the platelets were retained in the column, and there was massive production of both TXA_2 and PGI_2. Both these products were derived from arachidonic acid of platelet membrane origin. The evidence to support this conclusion is threefold.

First, when the HUVECs were preincubated with indomethacin, the PGI_2 response was not significantly different from that seen when untreated HUVECs were perfused with collagen-activated platelets. This suggests that HUVEC cyclooxygenase activity is not essential and that the substrates for the HUVEC PGI_2 synthetase in this experiment are lipid endoperoxides generated by and transferred from the platelets. These experiments were not strictly quantitative and furthermore do not reflect a physiological situation; rather, they are the result of pharmacological manipulation. Therefore, a second line of evidence was sought using isotopically labeled AA incorporated into both platelets and endothelium as an index of AA release and metabolism.

The passage of collagen-stimulated platelets labeled with $[^3H]$-AA did not cause the release of $[^{14}C]$-AA or its metabolites from the HUVEC column, suggesting that neither the proximity of aggregating platelets nor products released from them resulted in stimulation of endothelial membrane phospholipase. There was, however, the expected release of substantial $[^3H]$-AA metabolites from the activated platelets. During the early phase of the perfusion, these metabolites were shown to be identical to those produced by stimulated platelets alone on the basis of RP chromatography of the isotopically labeled products. There were three prominent metabolites: TXB_2, 12-HETE, and a third, assumed to be HHT.

After some minutes of perfusion there were additional peaks representing 6-keto-$PGF_{1\alpha}$, PGE_2 or PGD_2 and 15-HETE. These products have been reported to be produced by pulmonary ECs,[23] and represent the metabolism of either AA or cyclic endoperoxides transferred from the activated platelets. The production of 15-HETE by ECs could be important in the regulation of the inflammatory process locally in the endothelium. 15-HETE has been shown to regulate leukotriene biosynthesis in leukocytes, mast cells, and 12-lipoxygenase pathway in platelets.[24-26] In addition, 15-HETE enhances human neutrophil degranulation to PAF.[27] It would seem that generation of this product by ECs could be protective by several mechanisms. RIA of the fractions showed again the presence of TXB_2 and 6-keto-$PGF_{1\alpha}$, suggesting that the metabolism of recently incorporated isotopically labeled AA is at least representative of the metabolism of the nonlabeled pool.

Finally, the injection of putative agonists which are platelet-derived did not result in reproducible PGI_2 release. Pearson et al[7] showed that piglet aortic ECs responded to both ADP and ATP with PGI_2 release, the pattern of response being consistent with purinergic P_2 receptors. The lack of response to ADP and ATP seen in our experiments is in agreement with Acharya and MacIntyre[6] and Wecksler and colleagues[28] using HUVECs suggesting species or site specificity. Acharya and MacIntyre[6] and Test and Bang[9] recorded PGI_2 release induced by PAF. We were unable to confirm that this occurs as an acute response using concentrations up to 10^{-5} mol/L. Other platelet products with possible effects on EC PGI_2 release are α-granule proteins β-thromboglobulin,[29] and PDGF.[8] Any influence of these proteins may be exerted over a longer time course and there is no evidence for or against an important role for these proteins from our present experiments. However, basal PGI_2 release by piglet aortic endothelial cells in culture was identical whether the medium was supplemented with homologous serum or plasma-derived serum, suggesting that platelet release products do not exert a significant chronic effect.[30]

Thrombin regularly caused a striking but transient (despite continued exposure) release of PGI_2 with the development of tachyphylaxis. This phenomenon may be related to receptor occupancy and internalization or to inhibition of the peroxidase component of cyclooxygenase by its products[31] or by the accumulation of lipid peroxides due to lipoxygenase.[32] Our results confirm the phenomenon of transfer of AA metabolites from activated platelets to local ECs as shown by others[10-12] in less physiological models. They suggest that in the context of minor endothelial damage or desquamation, this is likely to be the only mechanism for EC PGI_2 release. In vivo experiments reported by Deckmyn and colleagues[33] support this conclusion. One explanation for the efficiency of the transfer of cyclic endoperoxides not being demonstrated by some workers[13-15] is the possible requirement of direct cell–cell contact.[11] This requirement would be readily met by the model we have used, one which mimics closely the dynamics of the microcirculation.[16] Clearly, the elaboration of thrombin in the event of more extensive tissue injury will contribute to PGI_2 release. Repeated stimulation, however, is likely to exhaust this contribution, and once again the system may rely on platelet arachidonate metabolites for continued PGI_2 production.

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REFERENCES

1. Moncada S, Korbut R, Bunting S, Vane JR: Prostacyclin is a circulating hormone. Nature (London) 273:767, 1978

2. Patrono C, Preston FE, Vermylen J: Platelet and vascular arachidonic acid metabolites: Can they help detect a tendency towards thrombosis? Br J Haematol 57:209, 1984

3. Moncada S, Vane JR: Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. N Engl J Med 300:1142, 1979

4. Goldsmith JC, Kisker CT: Thrombin-endothelial cell interactions: Critical importance of endothelial cell vessel of origin. Thromb Res 25:131, 1982

5. Pearson JD, Carleton JS, Hutchings A: Prostacyclin release stimulated by thrombin or bradykinin in porcine endothelial cells cultured from aorta and umbilical vein. Thromb Res 29:115, 1983

6. Acharya SB, MacIntyre DE: Platelet products and vascular PGI_2 production. Prostaglandins Leukotrienes Med 10:73, 1983

7. Pearson JD, Slakey LL, Gordon JL: Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells. Biochem J 214:273, 1983

8. Coughlin SR, Moskowitz MA, Zetter RB, Antoniades HN, Levine L: Platelet-dependent stimulation of prostacyclin synthesis by platelet-derived growth factor. Nature (London) 288:600, 1980

9. Test ST, Bang NU: Platelet-activating factor (1-*o*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) stimulates prostacyclin synthesis by cultured human endothelial cell. Thromb Haemost 46:269, 1981

10. Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ: Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest 66:979, 1980

11. Schafer AI, Crawford DD, Gimbrone MA: Unidirectional transfer of prostaglandin endoperoxides between platelets and endothelial cells. J Clin Invest 73:1105, 1984

12. Bunting SR, Gryglewski R, Moncada S, Vane JR: Arterial walls generate from prostaglandin endoperoxides a substance (prostaglandin x) which relaxes strips of mesenteric and coeliac arteries and inhibits platelet aggregation. Prostaglandins 12:897, 1976

13. Needleman P, Wyche A, Raz A: Platelet and blood vessel arachidonate metabolism and interactions. J Clin Invest 63:345, 1979

14. Hornstra G, Haddenman E, Don JA: Blood platelets do not provide endoperoxides for vascular prostacyclin production. Nature (London) 279:66, 1979

15. Baenziger NL, Becherer PR, Majerus PW: Characterisation of prostacyclin synthesis in cultured human arterial smooth muscle cells, venous endothelial cells and skin fibroblasts. Cell 16:967, 1979

16. Davies PF: Microcarrier culture of vascular endothelial cells on solid plastic beads. Exp Cell Res 134:267, 1981

17. Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest 52:2745, 1973

18. Engvall E, Ruoslahti E: Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer 20:1, 1977

19. Thornton SC, Mueller SN, Levine EM: Human endothelial cells: Use of heparin in cloning and long-term serial cultivation. Science 22:623, 1983

20. Vargas JR, Radomski M, Moncada S: The use of prostacyclin in the separation from plasma and washing of human platelets. Prostaglandins 32:929, 1982

21. Henke DC, Kolizan S, Eling TE: Analysis of leukotrienes, prostaglandins and other oxygenated metabolites of arachidonic acid by high-performance liquid chromatography. Anal Biochem 140:87, 1984

22. Ludlam CA, Cash JD: Studies on the liberation of β-thromboglobulin from human platelets *in vitro*. Br J Haematol 33:239, 1976

23. Johnson AR, Revtyak G, Campbell WB: Arachidonic acid metabolites and endothelial injury: Studies with cultures of human endothelial cells. Fed Proc 44:19, 1985

24. Vanderhoek JY, Bryant RW, Bailey JM: Inhibition of leukotriene biosynthesis by the leukocyte products 15-hydroxy-5,8,11,13-eicosatetraenoic acid. J Biol Chem 255:10064, 1980

25. Vanderhoek JY, Tare NS, Bailey JM, Goldstein AL, Pluznik DH: New role for 15-hydroxyeicosatetraenoic acid. J Biol Chem 257:12191, 1982

26. Vanderhoek JY, Bryant RW, Bailey JM: 15-hydroxy-5,8,11,13-eicosatetraenoic acid: A potent and selective inhibitor of platelet lipoxygenase. J Biol Chem 255:5996, 1980

27. O'Flaherty JT, Thomas MJ: Effect of 15-lipoxygenase-derived arachidonate metabolites on human neutrophil degranulation. Prostaglandins Leukotrienes Med 17:199, 1985

28. Wecksler BB, Ley WC, Jaffe AE: Stimulation of endothelial cell prostacyclin production by thrombin, trypsin and the ionophore A23187. J Clin Invest 62:923, 1978

29. Hope W, Martin TJ, Chesterman CN, Morgan FJ: Human

β-thromboglobulin inhibits PGI$_2$ production and binds to a specific site in bovine aortic endothelial cells. Nature (London) 282:210, 1979

30. Chesterman CN, Ager A, Gordon JL: Regulation of prostaglandin production and ectoenzyme activities in cultured aortic endothelial cells. J Cell Physiol 116:45, 1983

31. Egan RW, Paxton J, Kuehl FA Jr: Mechanism for irreversible self-deactivation of prostaglandin synthetase. J Biol Chem 251:7329, 1976

32. Siegel MI, McConnell RT, Abrahams SL, Porter NA, Cuatrecasas P: Regulation of arachidonate metabolism via lipoxygenase and cyclooxygenase by 12-HPETE, the product of human platelet lipoxygenase. Biochem Biophys Res Commun 89:1273, 1979

33. Deckmyn H, Van Houtte E, Verstraete M, Vermylen J: Manipulation of the local thromboxane and prostacyclin balance *in vivo* by the antithrombotic compounds dazoxiben, acetylsalicyclic acid and nafazatrom. Biochem Pharmacol 32:2757, 1983

Characterization of Fibrinogen Milano I: Amino Acid Exchange γ330Asp → Val Impairs Fibrin Polymerization

By P. Reber, M. Furlan, C. Rupp, M. Kehl, A. Henschen, P.M. Mannucci, and E.A. Beck

An abnormal fibrinogen was found in two asymptomatic members (father and daughter) of the same family, originating from northern Italy. Routine coagulation studies revealed prolonged thrombin and reptilase clotting times. Plasma fibrinogen levels, as determined by a functional assay, were markedly diminished, whereas the heat precipitation method indicated normal fibrinogen values. On the basis of these findings, a tentative diagnosis of dysfibrinogenemia was made, and according to the accepted nomenclature, this fibrinogen variant was called "fibrinogen Milano I." The time course of fibrinopeptide A and B release from fibrinogen Milano I was normal, but the aggregation of fibrin monomers was delayed. Two-dimensional electrophoresis of reduced variant fibrinogen chains showed a defective γ-chain with increased cathodic mobility. An abnormal electrophoretic mobility was observed also for the γ-chain remnants of fibrinogen fragments D_1 and D_2 derived from fibrinogen Milano I, whereas the charge anomaly was lost after a further digestion by plasmin to D_3, suggesting that the structure abnormality of this variant is situated in the region γ303-356. An abnormal peptide was isolated after cyanogen bromide cleavage of intact fibrinogen Milano I. This fragment spans from position γ311 to γ336. Amino acid analysis of the abnormal peptide showed the presence of valine and a diminished content of aspartic acid. Sequence analysis demonstrated an amino acid exchange Asp → Val in the γ-chain at position 330.

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"DYSFIBRINOGENEMIA" defines a defect of the fibrinogen molecule that is expressed by its functional behavior. Imperato and Dettori[1] described the first patient with congenital dysfibrinogenemia in 1958. Since then, reports on more than 120 kindreds or individuals with an abnormal fibrinogen have been published.[2] Abnormal fibrinogen variants are valuable models for studying the effect of a single amino acid substitution on the functional properties of this protein. The majority of abnormal fibrinogens, known until now, have been shown to be defective in their amino-terminal region, either within or adjacent to the fibrinopeptides A and B.[3] After removal of fibrinopeptides from normal fibrinogen, two pairs of binding sites, which are essential for polymerization, are exposed, ie, *N*-terminal sequences Gly-Pro-Arg- (α-chain) and Gly-His-Arg- (β-chain). These amino-termini of the fibrin monomer interact with as yet hypothetical "complementary" polymerization sites in the carboxy-terminal portions of the γ- and β-chains.[4-6] In this report, we describe a new inherited variant of fibrinogen, tentatively called fibrinogen Milano I. It exhibits a hitherto unpublished exchange of aspartic acid in position γ330 by valine, which seems to be responsible for delayed polymerization of fibrin monomers derived from this variant.

MATERIALS AND METHODS

Purification of fibrinogen. Fibrinogen was isolated from citrated plasma by affinity chromatography on fibrin monomer-Sepharose CL-2B.[7] Normal and abnormal fibrinogen molecules originating from the heterozygous carrier of the defective fibrinogen were not separated.

Determination of fibrinogen concentrations. Plasma levels of fibrinogen were measured by the functional assay of Clauss,[8] by the heat precipitation method of Schulz,[9] and by the Laurell immunoelectrophoresis technique.[10]

Measurement of fibrin aggregation. Fibrin aggregation was evaluated turbidimetrically: Fibrinogen (0.5 mg/mL) was preincubated with 0.1 mmol/L $CaCl_2$ for ten minutes at 37 °C. After addition of thrombin (bovine thrombin; Hoffmann-La Roche, Basel, Switzerland; 0.5 NIH U/mL final concentration), the increase in turbidity was measured in a thermostated photometer at 350 nm.

Fibrinopeptide release. The method of Kehl et al[11] for high-performance liquid chromatography (HPLC) analysis of fibrinopeptides was used with minor modifications. Fibrinogen solutions were dialyzed extensively against 0.15 mol/L ammonium acetate, pH 7.4, and diluted with the same buffer to 1 mg/mL. To 10 mL of dialyzed solution, $CaCl_2$ (0.1 mmol/L final concentration) and thrombin (0.1 NIH U/mL) were added and the sample incubated at 37 °C. The reaction was stopped by addition of 1 mL concentrated formic acid. The fibrin, which was completely soluble at the resulting pH of 2, was reprecipitated by neutralizing the solution with 25% NH_3 (final pH, 6.5). After centrifugation, the supernatant was lyophilized. The dry residue was dissolved in 100 μL 0.025 mol/L ammonium acetate, pH 6, and analyzed by reverse-phase HPLC. Chromatography on a Beckman (Fullerton, Calif) Ultrasphere-ODS RP-18 5μ (0.46 × 25 cm) column was performed at a flow rate of 1.5 mL/min with the following buffer system: solvent A, 0.025 mol/L ammonium acetate, pH 6.0 (H_3PO_4); solvent B, 50% acetonitrile (HPLC grade; Fluka, Buchs, Switzerland) in 0.05 mol/L ammonium acetate, pH 6.0; gradient, 18% to 28% B in 20 minutes.

Two-dimensional electrophoresis of fibrinogen Aα-, Bβ-, and γ-chains. For isoelectric focusing (IF) of the three fibrinogen chains, the method of Kuyas et al[12] was used with minor modifications. The cross-linking agent of the 7% polyacrylamide gels was 25% *N,N*-diallyltartardiamide (BDH, Poole, England). The gel buffer contained 8 mol/L desalted urea, 0.5% Triton X-100, 1.6% Pharmalyte (Pharmacia, Uppsala, Sweden), pH 4 to 6.5, and 1.6% Pharmalyte, pH 6.5 to 9, resulting in a pH gradient from 4 to 9.

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Polymerization was initiated by NH_4-peroxodisulfate. Before electrophoresis, fibrinogen solutions were exhaustively dialyzed against 0.05 mol/L Tris-HCl and 8 mol/L urea, pH 8.5, at 4 °C. The protein solutions were then incubated for 20 minutes at 37 °C after addition of dithiothreitol (0.1 mol/L final concentration; Calbiochem-Behring, La Jolla, Calif). About 75 μg of mercaptolysed fibrinogen was loaded onto cylindrical IF-gels (0.27 × 7 cm) that had been prerun for 30 minutes. Focusing was performed in a gel electrophoresis apparatus (Buchler Instruments, Fort Lee, NJ) at 10 °C during 16 hours. Cathode buffer was 10 mmol/L ethanolamine and anode buffer 10 mmol/L glutamic acid. Maximum current was limited to 0.4 mA per gel.

After isoelectric focusing, the gels were incubated in 0.075 mol/L sodium barbital/0.01 mol/L EDTA/2% sodium dodecyl sulphate (SDS), pH 8.6, placed on slab gels and covered with 1% agarose in electrophoresis buffer (0.075 mol/L sodium barbital, 0.01 mol/L EDTA, 0.2% SDS, pH 8.6). The 7% polyacrylamide slab gel (13 × 7.5 × 0.35 cm) contained 5% by weight of methylene-bisacrylamide per acrylamide in 6 mol/L urea, 0.1 mol/L Tris, 0.2% SDS, pH 7.4. Two-dimensional electrophoresis was performed with 50 mA per slab at 110 V for six hours. Proteins were visualized by staining the gels overnight with 0.02% Coomassie brilliant blue in methanol, acetic acid, and water (25:7.5:67.5).

Two-dimensional electrophoresis of plasmin-digested fibrinogen fragments D_1, D_2, and D_3. Normal plasma fibrinogen and fibrinogen Milano I were digested by plasmin in the presence of 10 mmol/L Ca^{2+}, and the resulting fragment D_1 isolated on lysine-agarose, as described elsewhere.[13] Fragment D_1 was dialyzed against 0.05 mol/L triethanolamine, 0.1 mol/L NaCl, 10 mmol/L ethyleneglycol-bis-(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.4. The dialyzed solution of fragment D_1 (2 mg/mL) was incubated for three hours at 37 °C with plasmin (0.3 U/mL), produced by activation of plasminogen (Kabi, Stockholm) with 20 units streptokinase (Kabi) per unit plasminogen. The reaction was stopped by addition of phenylmethanesulfonyl fluoride (Fluka). The resulting mixture of fragments D_1, D_2, and D_3 was subjected to mercaptolysis and electrophoresed as described.

Cyanogen bromide cleavage of intact fibrinogen. Ten milligrams lyophilized fibrinogen was dissolved in 1 mL 10% (wt/vol) cyanogen bromide (CNBr; Fluka) in 70% formic acid. After incubation for two hours at room temperature, 10 mL distilled water was added. The solution was concentrated in a rotary evaporator and injected onto a Vydac TPRP-10 C-18 (Chrompack, Middelburg, Netherlands) reverse-phase HPLC column (0.6 × 25 cm).[14] The following buffer system was used: solvent A, 0.1% trifluoroacetic acid (TFA; Fluka) in water; solvent B, 0.1% TFA in acetonitrile; gradient, 5% to 50% B in 100 minutes; flow rate, 5 mL/min. The peptides were detected at 206 nm. The eluate, represented by the shaded area in Fig 4, was collected, mercaptolysed, and carboxymethylated.[15] The resulting peptide mixtures were fractionated on a Vydac TPRP-10 column as described earlier, using a gradient of 10% to 50% B in 60 minutes.

RESULTS

Case report. In 1980, a girl from northern Italy and her father were found by routine analysis to exhibit a blood coagulation defect in the absence of either bleeding or thrombosis. Results of laboratory investigations (Table 1) were compatible with congenital dysfibrinogenemia. In the following, purified fibrinogen, obtained from the plasma of the father, will be denoted as "fibrinogen Milano I," taking into consideration that it contained an unknown proportion of normal fibrinogen.

Table 1. Relevant Results of Laboratory Tests

Test	Dysfibrinogenemia		Normal Range
	Father	Daughter	
Clotting time (sec)			
Thrombin	21.0	21.4	11–14
Reptilase	24.7	21.4	14–15
Fibrinogen concentration (g/l)			
Clauss (functional assay)	0.4	0.5	1.5–3
Schulz (heat precipitation)	3.0	2.1	1.5–3
Laurell (immunologic assay)	2.4	2.0	1.5–3
Bleeding time (min)	3:45	4:10	<7

Fibrinopeptide release and fibrin monomer aggregation. The kinetics of fibrinopeptide cleavage by thrombin from the normal and variant fibrinogen is shown in Fig 1. The amounts of fibrinopeptides A and B released from normal fibrinogen within 60 minutes were taken as 100%. Both fibrinopeptides from normal and variant fibrinogen were released at similar rates, indicating that the initial enzymatic reaction in the conversion of fibrinogen to fibrin is not delayed with fibrinogen Milano I. In the presence of 0.1 mmol/L Ca^{2+}, fibrin monomers derived from Milano I showed a retarded aggregation with a t_{lag} of 110 seconds as compared with a t_{lag} of 13 seconds for normal fibrin.

Two-dimensional electrophoresis of fibrinogen Aα-, Bβ-, and γ-chains. Two-dimensional electrophoresis (Fig 2) showed the characteristic charge heterogeneity of the three reduced chains of normal fibrinogen. The Aα- and Bβ-chains of fibrinogen Milano I did not differ from those of normal fibrinogen, while the γ-chain of the abnormal fibrinogen exhibited an additional band, shifted to the cathode, thus suggesting that the defect might be located in the γ-chain and that an exchange of an acidic amino acid against a

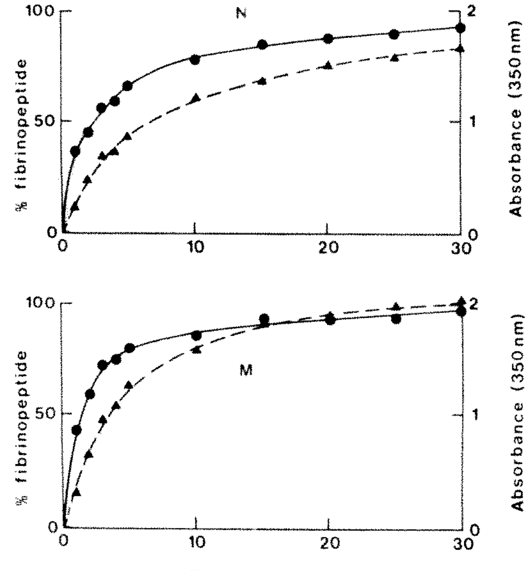

Fig 1. Fibrinopeptide release and fibrin aggregation curves of normal plasma fibrinogen (N) and fibrinogen Milano I (M). ——, release of fibrinopeptide A; ____, release of fibrinopeptide B; ……., fibrin aggregation curve, measured at 350 nm.

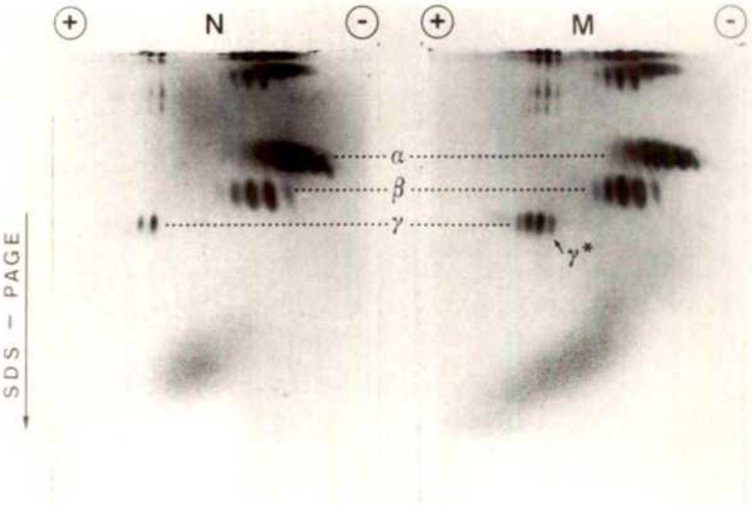

Fig 2. **Two-dimensional electrophoresis slab gels of reduced fibrinogen chains. The abnormally migrating γ-chain band of fibrinogen Milano I is marked with an asterisk.**

neutral or of a neutral amino acid against a basic one was present in fibrinogen Milano I.

Two-dimensional electrophoresis of reduced fragments D_1, D_2, and D_3. The resolution of the polypeptide chains obtained by disulfide reduction of the plasmic degradation products D_1, D_2, and D_3 is shown in Fig 3. All three fragments D, derived from normal fibrinogen, contained the same β-chain remnant having a molecular weight (mol wt) of 42,000. The main γ-chain remnants of the fragments D_1, D_2, and D_3 had apparent mol wt of 39,000, 34,000, and 25,000, respectively. Only faintly stained bands of the αD-chain (mol wt 10,000) were observed at the bottom of the gels. Differences were noted in charge of the γ-chain remnants, derived from normal and abnormal fragments D_1 and D_2. In fibrinogen Milano I, the γD_2-remnants are obviously duplicated, the spots of the defective remnants showing a significant shift in charge. These differences disappeared in the γ-chain remnant of D_3, suggesting that the abnormal peptide had been cleaved off by plasmin during conversion of D_2 to D_3.

Cyanogen bromide cleavage of intact fibrinogen. The HPLC elution pattern of the CNBr-cleaved fibrinogen Milano I showed a reproducible difference in the peaks, with the retention times between 62.5 and 64.5 minutes from the corresponding pattern of normal fibrinogen (Fig 4). The relevant peaks of the normal and abnormal fibrinogen, indicated by the shaded area, were collected, mercaptolysed, carboxymethylated, and rechromatographed by HPLC (Fig 5). The first four N-terminal amino acids of the peptide eluting with a retention time of 28.9 minutes were Gln-Phe-Ser-Thr-, ie, QFST. Thus this peptide corresponds to the cyanogen bromide fragment γ311-336.[16] The HPLC diagrams of the material, derived from fibrinogen Milano I, showed a diminished normal QFST peak and an abnormal QFST* peak, with a retention time of 31.6 minutes. The two QFST peptides were collected, and amino acid analysis and sequence analysis were performed. Results of the amino acid analysis are given in Table 2. Sequence analysis of the QFST* peak (Fig 6) indicated the presence of valine instead of aspartic acid in position γ330. In addition to the QFST fragments, two other peptides were eluted by rechromatography, with *N*-terminal amino acids NKCH, a γ-chain fragment, and DLGT, a peptide derived from the α-chain.

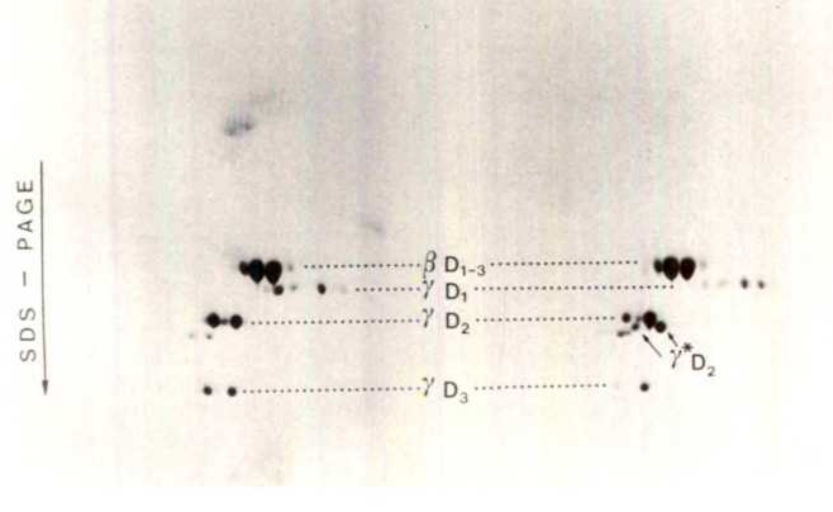

Fig 3. **Two-dimensional electrophoresis of a mixture of mercaptolysed fibrinogen fragments D_1, D_2, and D_3. Two additional γ-chain spots in the Milano I gel are denoted with an asterisk.**

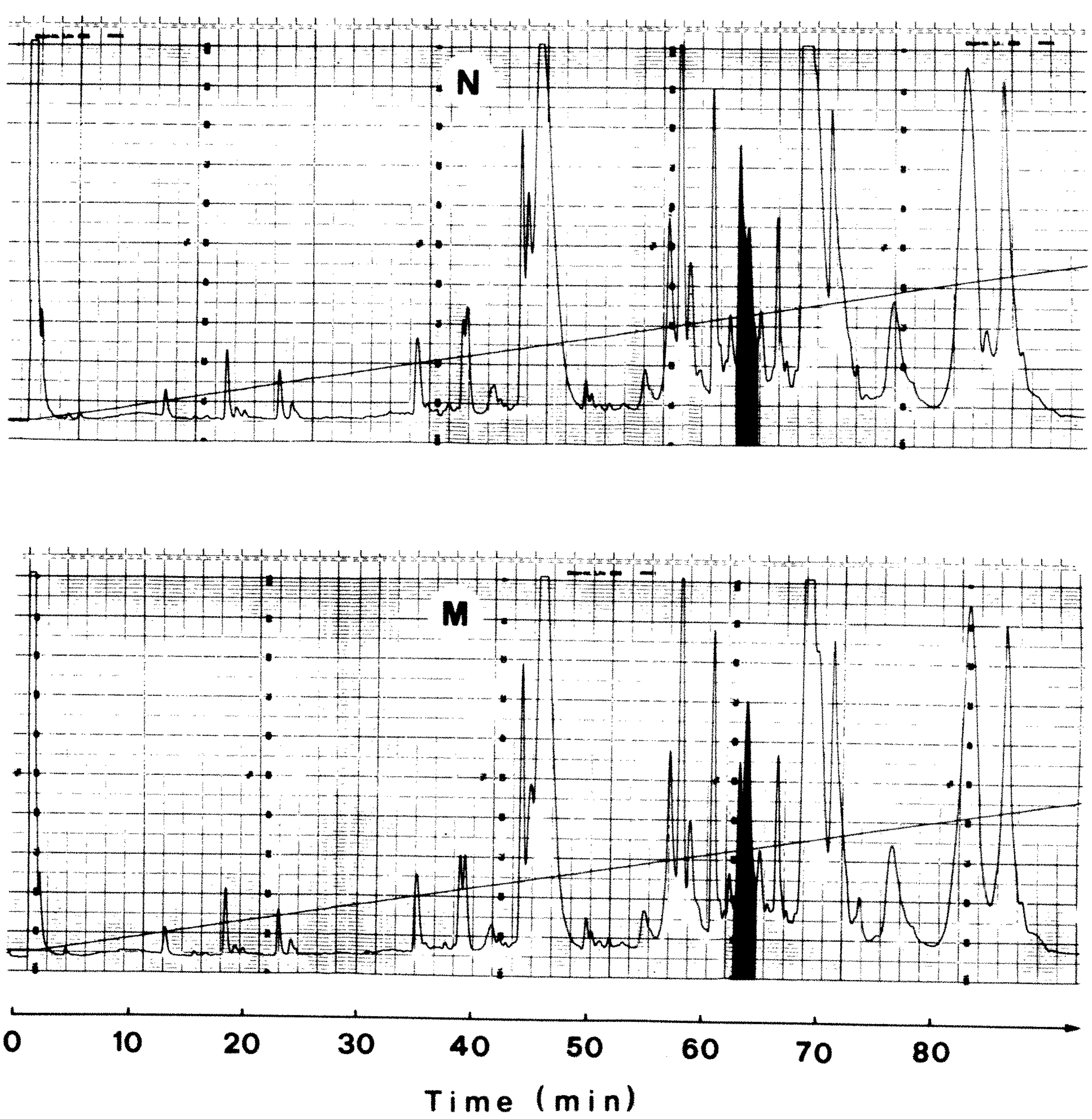

Fig 4. HPLC diagram of cyanogen bromide-cleaved intact fibrinogen. The peaks of the shaded area were collected, reduced, and carboxymethylated. HPLC conditions: Vydac TPRP-10 (0.6 × 25 cm); solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 5% to 50% B in 100 minutes; flow rate, 5 mL/min; detection, 206 nm. N, normal fibrinogen; M, fibrinogen Milano I.

DISCUSSION

We describe a family with two members heterozygous for an abnormal fibrinogen, denoted as fibrinogen Milano I, according to the accepted nomenclature. Fibrinogen determination resulted in a striking difference between the values obtained by the functional test and heat precipitation or electroimmunoassay. The thrombin and reptilase clotting times of both plasmas were markedly prolonged.

Aggregation of fibrin derived from thrombin-treated dysfibrinogen was delayed, whereas fibrinopeptide release was normal, thus indicating that the defect should not be sought for in the amino-terminus of the molecule.

Two-dimensional electrophoresis of reduced D_1, D_2, and D_3 fragments demonstrated a dramatic shift in the isoelectric points of the normal γ-chain remnants observed after conversion of D_1 to D_2. Their relative mobilities suggested that the carboxy-terminal amino acid of γD_2 is most likely 356Lys. The sequence γ357Ala-411Val is characterized by an excess of six basic amino acids. As a consequence of its removal by plasmin, the isoelectric point of γD_2 becomes considerably more acidic than that of γD_1. The carboxy terminus of the γD_3 remnant presumably ends at 302Lys. The peptide γ303Phe-356Lys contains the same number of acidic and basic amino acids. Therefore, no shift in isoelectric point resulted when normal γD_2 was digested by plasmin to γD_3.

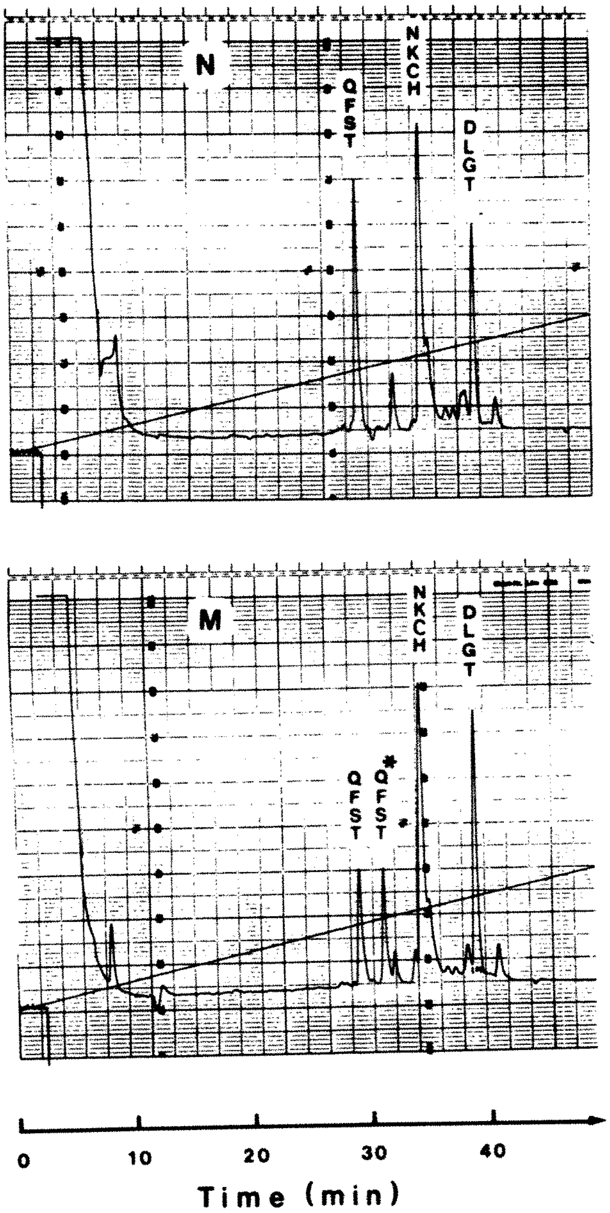

Fig 5. HPLC diagram of the reduced and carboxymethylated peptides, described in Fig 4. HPLC conditions: Vydac TPRP-10 (0.6 × 25 cm); solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient, 10% to 50% B in 60 minutes; flow rate, 5 mL/min; detection, 206 nm. Amino acid and sequence analyses were performed with normal QFST and abnormal QFST* material, derived from fibrinogen Milano I (Table 2). N, normal fibrinogen, M, fibrinogen Milano I.

The γD_2 chain of fibrinogen Milano I produced, on two-dimensional electrophoresis slabs, two additional spots, shifted toward the cathode. These additional bands disappeared when γD_2 remnant was further degraded by plasmin, thus implying that the defect is located between γ-chain residues 303Phe and 356Lys. We propose two-dimensional electrophoresis of reduced plasmin fragments D_1, D_2, and D_3 as a simple screening method in investigating molecular defects in the carboxy terminal domain of the fibrinogen γ-chain.

Table 2. Amino Acid Composition of Two HPLC Peaks From Fibrinogen Milano I

Amino Acid	QFST Peptide γ311-336		QFST* Peptide γ311-336
	Theoretical	Normal Peak	(Abnormal Peak)
Scm-Cys	1	0.6	0.6
Asp/Asn	7	6.8	5.6
Thr	1	0.9	0.9
Ser	2	2.0	2.2
Met	1	0.8	0.8
Glu/Gln	4	4.3	4.3
Gly	3	3.3	3.3
Ala	1	1.3	1.2
Val	—	—	1.0
Phe	2	1.9	1.7
Lys	1	1.2	1.3
Trp	3	ND	ND

Peptides were eluted with retention times of 28.9 minutes for normal and 31.6 minutes for variant fibrinogen (Fig 5). Scm-Cys, carboxymethylcysteine; ND, not determined.

After CNBr digestion of intact fibrinogen Milano I, an abnormal peak was isolated by HPLC. This peak contained γ-chain peptides starting with the sequences QFST and NKCH, linked together by the disulfide bridge γ326Cys-γ339Cys and the α-chain fragment DLGT. After mercaptolysis and carboxymethylation of this material, the two QFST species were isolated by HPLC. Amino acid analysis of the abnormal QFST* peak showed a diminished aspartic acid content. It contained 1 mol of valine, while the equivalent peptide from the patient's normal fibrinogen molecules lacks this amino acid residue (Table 2). The sequence analysis of the abnormal QFST* fragment demonstrated the amino acid substitution γ330Asp → Val. Chung et al[17] reported that the codon for amino acid γ330 of human fibrinogen is GAT. This codon probably has been changed to GTT in fibrinogen Milano I. Comparison of the human QFST sequence with the corresponding sequences of rat[18] and lamprey[19] fibrinogen showed that the amino acid exchange took place in an evolutionary highly conserved region of the molecule (Fig 6). In rat fibrinogen, the γ-chain differs by only one amino acid in the QFST peptide from that of human fibrinogen, the amino-terminal glutamine being replaced by a histidine residue.[18] The exchange of γ330Asp by Val obviously affects the polymerization behavior of fibrin monomers derived from fibrinogen Milano I: after thrombin addition to the abnormal fibrinogen preparation, the onset of the turbidity increase was delayed.

Other authors have suggested that the polymerization site is located in the carboxy terminus of the γ-chain. Thus the peptides containing amino acid sequence γ374-411 were

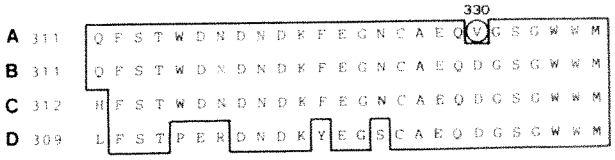
330
A 311 Q F S T W D N D N D K F E G N C A E Q V G S G W W M
B 311 Q F S T W D N D N D K F E G N C A E Q D G S G W W M
C 312 H F S T W D N D N D K F E G N C A E Q D G S G W W M
D 309 L F S T P E R D N D K Y E G S C A E Q D G S G W W M

Fig 6. Sequence comparison of the QFST peptides of (B) normal human,[16] (C) rat,[18] and (D) lamprey[19] fibrinogen with the respective region of (A) fibrinogen Milano I.

shown to inhibit the polymerization of fibrin monomers.[6] Furthermore, fragment D_1, having an intact carboxy terminus of the γ-chain, was an efficient polymerization inhibitor, whereas fragment D_2, lacking the carboxy-terminal peptide γ357-411, was not inhibitory.[20] Our data indicate that specific amino acid residues, located beyond the tentative polymerization domain, might also be involved in the polymerization process. The cross-linking sites in the γ-chain were identified as Gln398 and Lys406.[21] We observed complete dimerization of γ-chains in fibrin monomers derived from fibrinogen Milano I (data not shown). The carboxy-terminal portion of the γ-chain contains binding sites for platelets.[22] Furthermore, a high-affinity calcium binding site was localized in the fragment γ311-336[23]; this γ-chain peptide showed a strong homology with calmodulin.[24] Studies of fibrinogen Milano I interactions with calcium and platelets are in progress.

In conclusion, a new molecular defect was identified in a fibrinogen variant called fibrinogen Milano I. Our results demonstrate that the aspartic acid in position γ330 is indispensable for normal polymerization of fibrin monomers.

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REFERENCES

1. Imperato C, Dettori AG: Ipofibrinogenemia congenita con fibrinoastenia. Helv Paediatr Acta 13:380, 1958

2. Rupp C, Beck EA: Congenital dysfibrinogenemia, in Beck EA, Furlan M (eds): Variants of Human Fibrinogen. Berne, Hans Huber, 1984, p 65

3. Henschen A, Kehl M, Southan C: Genetically abnormal fibrinogens—Strategies for structure elucidation, including fibrinopeptide analysis, in Beck EA, Furlan M (eds): Variants of Human Fibrinogen. Berne, Hans Huber, 1984, p 273

4. Kudryk BJ, Collen D, Woods KR, Blombäck B: Evidence for localization of polymerization sites in fibrinogen. J Biol Chem 249:3322, 1974

5. Olexa SA, Budzynski AZ: Evidence for four different polymerization sites involved in human fibrin formation. Proc Natl Acad Sci USA 77:1374, 1980

6. Olexa SA, Budzynski AZ: Localization of a fibrin polymerization site. J Biol Chem 256:3544, 1981

7. Furlan M, Rupp C, Beck EA, Svendsen L: Effect of calcium and synthetic peptides on fibrin polymerization. Thromb Haemost 47:118, 1982

8. Clauss A: Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. Acta Haematol (Basel) 17:237, 1957

9. Schulz FH: Eine einfache Bewertungsmethode von Leberparenchymschäden (volumetrische Fibrinbestimmung). Acta Hepat 3:306, 1955

10. Laurell C-B: Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal Biochem 15:45, 1966

11. Kehl M, Lottspeich F, Henschen A: Analysis of human fibrinopeptides by high-performance liquid chromatography. Hoppe-Seyler's Z Physiol Chem 362:1661, 1981

12. Kuyas C, Haeberli A, Straub PW: A subfraction of human fibrinogen with high sialic acid content and elongated γ-chains. J Biol Chem 257:1107, 1982

13. Rupp C, Sievi R, Furlan M: Fractionation of plasmic fibrinogen digest on lysine-agarose. Isolation of two fragments D, fragment E and simultaneous removal of plasmin. Thromb Res 27:117, 1982

14. Kehl M, Lottspeich F, Henschen A: High performance liquid chromatography as applied to the studies of the fibrinogen structure, in Molnar I (ed): Practical Aspects of Modern HPLC. Berlin, Walter de Gruyter, 1982, p 137

15. Henschen A, Edman P: Large scale preparation of S-carboxymethylated chains of human fibrin and fibrinogen and the occurrence of γ-chain variants. Biochim Biophys Acta 263:351, 1972

16. Lottspeich F, Henschen A: Amino acid sequence of human fibrin. Preliminary note on the completion of the γ-chain sequence. Hoppe-Seyler's Z Physiol Chem 358:935, 1977

17. Chung DW, Chan W-Y, Davie EW: Characterization of a complementary deoxyribonucleic acid coding for the γ chain of human fibrinogen. Biochemistry 22:3250, 1983

18. Crabtree GR, Kant JA: Organization of the rat γ-fibrinogen gene: Alternative mRNA splice patterns produce the γA and γB (γ') chains of fibrinogen. Cell 31:159, 1982

19. Strong DD, Moore M, Cottrell BA, Bohonus VL, Pontes M, Evans B, Riley M, Doolittle RF: Lamprey fibrinogen γ chain: Cloning, cDNA sequencing, and general characterization. Biochemistry 24:92, 1985

20. Nieuwenhuizen W, Voskuilen M, Vermond A, Haverkate F, Hermans J: A fibrinogen fragment D (D intermediate) with calcium binding but without anticlotting properties. Biochim Biophys Acta 707:190, 1982

21. Chen R, Doolittle RF: Cross-linking sites in human and bovine fibrin. Biochemistry 10:4486, 1971

22. Kloczewiak M, Timmons S, Lukas TJ, Hawiger J: Platelet receptor recognition site on human fibrinogen. Synthesis and structure-function relationship of peptides corresponding to the carboxy-terminal segment of the γ-chain. Biochemistry 23:1767, 1984

23. Dang CV, Ebert RF, Bell WR: Localization of a fibrinogen calcium binding site between γ-subunit position 311 and 336 by terbium fluorescence. J Biol Chem 260:9713, 1985

24. Henschen A, Lottspeich F, Kehl M, Southan C: Covalent structure of fibrinogen. Ann NY Acad Sci 408:28, 1983

Expression of the Leukocyte Functional Molecule (LFA-1) on Mouse Platelets

By Peter J. McCaffery and Michael V. Berridge

Platelet involvement in adhesion, hemostasis, and immune adherence is mediated by functionally associated cell surface molecules. Platelets are also involved in cytolytic reactions, but little is known about the mechanisms or biologic significance of these processes. To further investigate cell surface molecules concerned with platelet function, antisera against mouse platelets, thymocytes, and macrophages and monoclonal antibodies against Mac-1 (complement receptor type 3) and leukocyte function-associated glycoprotein type 1 (LFA-1) were used to demonstrate LFA-1-like molecules on mouse platelets. The α subunits of platelet and thymocyte LFA-1 showed identical electrophoretic mobility but differed significantly from the α subunit of macrophage Mac-1. Peptide mapping demonstrated the identity of the β subunits of these three molecules but showed that the α subunit of Mac-1 was distinct from the α subunits of platelet and thymocyte LFA-1. Platelet LFA-1, as demonstrated by surface iodination with lactoperoxidase and by labeling sialic acid residues with sodium borohydride, was not a major component of the platelet membrane. The functional significance of LFA-1 on mouse platelets has yet to be demonstrated, monoclonal antibodies against LFA-1 having little effect on adenosine diphosphate-induced platelet aggregation and immune adherence. In contrast, although Mac-1 could not be demonstrated on mouse platelets in immunoprecipitation studies, its presence was clearly demonstrated by low levels of antibody binding in enzyme-linked immunosorbent assays and by the ability of M1/70 monoclonal antibody to inhibit platelet immune adherence. Human platelets, which are inactive in immune adherence assays, are shown to lack LFA-1 and Mac-1.

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THE PLATELET cell surface has been the subject of several studies, the main proteins being numerically denoted as I to V according to their electrophoretic mobilities.[1,2] Several platelet membrane proteins play important roles in platelet adherence and agglutination,[3-5] but the functions of other platelet cell surface proteins have still to be elucidated.

A family of glycoproteins found on many hematopoietic cells has recently been demonstrated to have a functional role in cell-cell adhesion in both mice and humans. These glycoproteins, the leukocyte function-associated molecules (LFA-1),[6] and the type three complement receptor Mac-1[7] are both heterodimers. They share an identical β subunit of 95,000 molecular weight (mol wt) but differ in the structure of their α subunits, which have mol wts of 180,000 and 170,000, respectively.[8,9] The α subunits can also be distinguished by their isoelectric points and by peptide patterns produced by partial enzymic digestion.

Monoclonal antibodies (MoAb) specific for the α subunit of Mac-1 prevent the adherence of inactivated C3b-coated particles to macrophages, granulocytes, and natural killer (NK) cells by blocking the membrane receptor for inactivated C3b.[10] LFA-1 has a much wider distribution on hematopoietic cells, being present on both myeloid and lymphoid cells where it is involved in the adhesion of cytotoxic lymphocytes to target cells and in mixed lymphocyte responses.[11] The biologic importance of LFA-1 and Mac-1 has also been demonstrated by functional defects in leukocytes that occur as a result of the absence of these glycoproteins.[12,13]

In the mouse, the cellular distribution of LFA-1 can now be extended to include platelets. Thus, we have used several MoAb and heterologous antisera to demonstrate LFA-1-like molecules on platelets. In particular, we have determined the relationship of platelet LFA-1 to previously classified platelet glycoproteins and to LFA-1 and Mac-1 on mouse thymocytes and macrophages. Although platelet LFA-1 does not correspond to one of the major platelet glycoproteins, its relationship to LFA-1 on thymocytes and Mac-1 on macrophages is clearly demonstrated.

MATERIALS AND METHODS

Cell preparation and absorptions. CBA, CBA/N, and (CBAxC57 BL/6)F_1 mice were bred at Wellington Clinical School. Platelets and thymocytes were prepared as described previously[14] from CBA or F_1 cross mice. Macrophages were isolated from peritoneal cells of CBA or CBA/N mice chronically infected with *Mesocestoides corti* larvae. Repeated differential sedimentation at unit gravity gave macrophages of greater than 96% purity. Absorptions were carried out at 4 °C for 45 minutes.

MoAb and antisera. M1/70, I21/7.7, and I41/14.1 were obtained from Dr I. Trowbridge (Salk Institute, La Jolla, Calif) and M17/5.1.9 and TS1/18 from Dr T.A. Springer (Dana-Farber Cancer Institute, Boston). EDU-3 was obtained from Dr R. Vilella (Hospital Clinico y Povincial, Barcelona, Spain), whereas 1G3 and 7G6 were derived in this laboratory by fusion of SP2/0 plasmacytoma cells with spleen cells from a rat inoculated with mouse platelets. Antithymocyte serum (ATS), antimacrophage serum (AMS), and antiplatelet serum (APS) were prepared as described previously[14] and were absorbed with an equal volume of washed erythrocytes. Molecular weights of the α-chain of Mac-1 and LFA-1 have shown consistently lower values than those originally described,[6,7] and we have designated them 160,000 and 170,000 mol wts throughout this paper.

Antibody-binding assays. Immunofluorescence was determined using fresh cells and fluorescently labeled affinity-purified IgG mouse antirat IgG, IgG rabbit antimouse IgG (Miles-Yeda, Rehovot, Israel), or IgG goat antirabbit IgG (Behringwerke, Marburg, FRG). Solid-phase enzyme-linked immunosorbent assays (ELISA) used glutaraldehyde-fixed platelets[15] and a 1:800 dilution of peroxidase-conjugated IgG rabbit antirat IgG (Amersham Corp, Sydney,

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Australia). Peroxidase substrate (0.1% o-phenylaminediamine, 0.012% H_2O_2 in 0.025 mol/L citrate-phosphate buffer, pH 4.7) was added and the reaction terminated after 10 minutes by adding an equal volume of 4 N H_2SO_4. Microtiter plates were read in an automated microplate reader (Dynatech, Singapore) at 490 nm using a reference wavelength of 410 nm.

Cell surface labeling and immunoprecipitation. Cells were labeled either with ^{125}I-sodium iodide (Amersham) by the glucose oxidase modification[16] of the lactoperoxidase technique[17] or with ^{3}H by mild oxidation of sialic acid residues followed by reduction with NaB^3H_4.[18] The radiolabeled cells were solubilized with 1% NP40 in 50 mmol/L Tris-HCl buffer, pH 7.2, containing 1 mmol/L phenylmethylsulphonyl fluoride and 0.3 U/mL aprotinin (Sigma Chemical Co, St Louis). Following two preabsorptions with 0.2 vol of 10% vol/vol *Staphylococcus aureus* Cowan strain 1 (SaC1), the cell lysates were treated with either antiserum or MoAb followed by rabbit antirat IgG (Miles-Yeda) second antibody. The resulting immune complexes were immunoprecipitated with SaC1, washed, and boiled for six minutes in sodium dodecyl sulfate (SDS) sample buffer (0.1 mol/L Tris-HCl, 2% SDS) with 5% mercaptoethanol unless otherwise stated. SaC1 was removed by centrifugation and the supernatants electrophoresed on 6% to 12.5% gradient SDS-polyacrylamide gels after the method of Laemmli.[19] Autoradiography was performed using intensifying screens.[20] For immunoprecipitation with ^{3}H-labeled material the polyacrylamide gels were treated with 20% 2,5-diphenyloxazole in acetic acid[21] and were exposed against preflashed films.[22] Peptide mapping was performed as described by Cleveland et al,[23] protein samples in gel slices being digested with varying quantities of V8-protease (Miles-Yeda) while running them through a 4.5% polyacrylamide stacking gel. Peptide fragments were separated by electrophoresis through a 15% acrylamide-resolving gel. Molecular weight markers were from Pharmacia Fine Chemicals (Uppsala, Sweden) and from Behring.

Immune adherence assay. Immune adherence was determined using platelet-dependent agglutination of antibody-coated sheep red blood cells (EA) in the presence of mouse complement. Platelet-rich plasma (PRP, 0.5 to 1.0 × 10^9 platelets/mL) was prepared from cardiac blood collected into siliconized tubes containing trisodium citrate as an anticoagulant. Following centrifugation at 200 g for ten minutes, PRP was removed and the remaining suspension centrifuged at 1,200 g to produce platelet-poor plasma (PPP). The platelet concentration was adjusted with PPP. Platelets were mixed with phosphate-buffered saline (PBS), or antibody (25 μL final volume) prior to the addition of erythrocytes or EA (25 μL, 1% vol/vol). EA were prepared by coating sheep red blood cells (2% vol/vol) with subagglutinating concentrations of rabbit hemolysin (Wellcome Reagents, Beckenham, England). Microtiter plates were agitated gently at 20-minute intervals and agglutination assessed at 60 to 90 minutes. The dependence of agglutination on complement was verified using purified platelets and fresh mouse serum or plasma.

Platelet aggregation. Cardiac blood was used to prepare PRP and PPP as previously described. Aggregation studies were performed at 37 °C under continuous stirring in a Corning EEL-169 platelet aggregometer. PRP (400 μL) was mixed with antiserum or hybridoma supernatant (50 μL) and aggregation initiated by the addition of 50 μl of 10 mmol/L adenosine diphosphate (ADP) in saline.

RESULTS

Binding of MoAb against LFA-1 and Mac-1 to mouse platelets. The derivation, specificity, and platelet-binding properties of antibodies used in this study are summarized in Table 1. Whereas the LFA-1–specific MoAb I21/7.7, and M17/5.1.9 bound weakly to mouse platelets, M1/70 against Mac-1 showed no detectable binding. MoAb IG3 against a platelet glycoprotein of mol wt 210,000 showed a similar binding pattern to LFA-1 antibodies, whereas EDU-3, a MoAb against the human platelet IIb/IIIa glycoprotein complex, bound weakly to a minor population of mouse platelets. In contrast, AP-IgG that had been extensively absorbed with thymus, brain, and peritoneal cells and was specific for the stem cell-megakaryocyte-platelet lineage[27] bound strongly to all platelets from both mouse and human species. Neither mouse or human LFA-1 nor Mac-1 MoAb bound significantly to human platelets. The binding of LFA-1 MoAb to glutaraldehyde-fixed mouse platelets was confirmed by ELISA (Fig 1). In contrast to results obtained with immunofluorescence, ELISA assays showed the presence of Mac-1 on mouse platelets, antibody binding being significantly above background and titrateable.

Effect of platelet absorption on the ability of ATS to immunoprecipitate T200 and LFA-1 family molecules from macrophages. Antiserum against mouse thymocytes immunoprecipitates T200 family and LFA-1 family molecules from ^{125}I-labeled macrophages (Fig 2, lane A3). T200 family molecules, which express leukocyte common antigens, occur in several different molecular forms on different hemato-

Table 1. Binding of MoAb Against Mac-1 and LFA-1 to Platelets

Antibody	Specificity	Mol Wt of Reduced Mouse Protein	Ig Subclass	Source Reference	Fluorescence Intensity* (Percentage Positive)	
					Mouse	Human
NSI†	—	—	—	—	−(0)	−(0)
M1/70	Mac-1 (α subunit)	160,000	rat IgG2b	7	−(0)	−(0)
I21/7.7	mouse LFA-1 (α subunit)	170,000	rat IgG	24	+(100)	−(0)
M17/5.1.9	mouse LFA-1 (α subunit)	170,000	rat IgG	25	+(100)	−(0)
TS1/18	human LFA-1 (α subunit)	—	mouse IgG	25	ND	−(0)
IG3	—	210,000	rat IgG	—	+(100)	−(0)
EDU-3	IIb/IIIa	100,000/112,000	mouse IgG2a	26	+(2-8)	+ + + +(100)
AP-IgG/abs	IIb/IIIa	100,000/112,000	rabbit IgG	27	+ + + +(100)	+ + +(100)

Abbreviation: ND, not determined.

*Fluoroscence intensity was arbitrarily rated from weakly positive (+) to strongly positive (+ + + +).

†NSI plasmacytoma culture supernatant was used as a control.

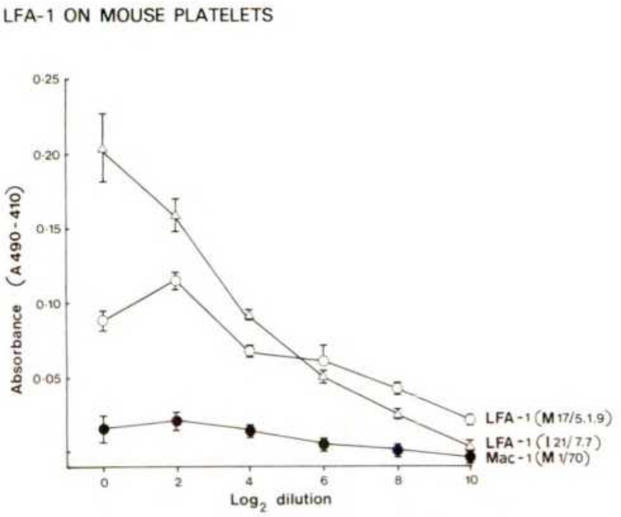

Fig 1. Binding of MoAb against LFA-1 and Mac-1 to mouse platelets: I21/7.7, ○; M17/5.1.9, △; M1/70, ●.

poietic cells.[28] The T200 identity of the 188,000–mol wt protein on macrophages was established using the MoAb I41/14.1 (Dr I. Trowbridge, personal communication, 1982) (Fig 2A, lane 1). The 160,000– and 97,000–mol wt bands were shown to be Mac-1 by preclearing experiments with MoAb M1/70 (results not shown). Absorption of ATS with platelets selectively removed antibodies immunoprecipitating Mac-1 but did not reduce immunoprecipitation of the macrophage T200 band (Fig 2A, lane 2). In contrast to the results obtained with ATS, neither platelet nor thymocyte absorption reduced the ability of AMS to immunoprecipitate Mac-1 from ^{125}I-labeled macrophages (Fig 2B, lanes 2 to 4).

ATS, AMS, and I21/7.7 immunoprecipitate LFA-1–like proteins from ^{125}I-labeled platelets. The major iodinated proteins immunoprecipitated from surface-labeled platelets by ATS and AMS have mol wts of 170,000 and 97,000 daltons (Fig 3, lanes 4 and 5). These proteins are of similar mol wt to those immunoprecipitated with I21/7.7 MoAb (Fig 3, lane 3), indicating the presence of LFA-1–like molecules on platelets. The relative intensities of the 170,000– and 97,000–mol wt bands differed between these immunoprecipitates. In contrast, M1/70 showed no detectable immunoprecipitable products from ^{125}I-labeled platelets (Fig 3, lane 2). Under reducing conditions, antiplatelet serum immunoprecipitated major bands of 150,000, 138,000, and a broad band of 100,000 to 112,000 mol wt (Fig 3, lane 1), consistent with their being platelet glycoproteins IIa, Ib, and IIb/IIIa respectively.[1] These results support the conclusions drawn from Fig 2 that the T200 family antigens recognized by ATS are not represented on platelets even though certain T200 MoAb have been shown previously to bind to platelets.[27]

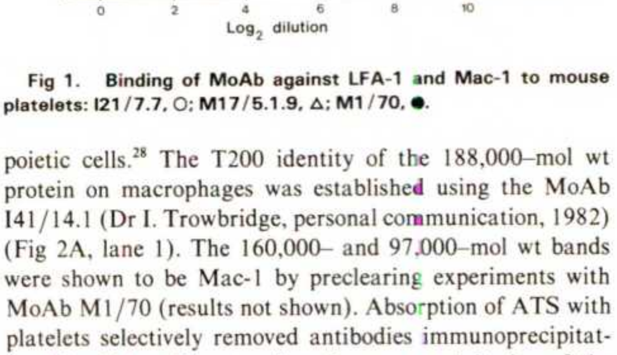

Fig 2. Absorption of antibodies against LFA-1 family molecules in ATS by platelets. Detergent lysates of macrophages surface labeled with ^{125}I were immunoprecipitated with (A) 1, I41/14.1 against T200; 2, ATS absorbed twice with platelets; 3, ATS; 4, NSI control hybridoma supernatant; and (B) 1, control rabbit serum; 2, AMS absorbed twice with thymocytes; 3, AMS absorbed twice with an equal volume of platelets; 4, AMS; 5, M1/70 against Mac-1. Immunoprecipitates were solubilized, electrophoresed on 6% to 12.5% gradient polyacrylamide gels, dried, and autoradiographed.

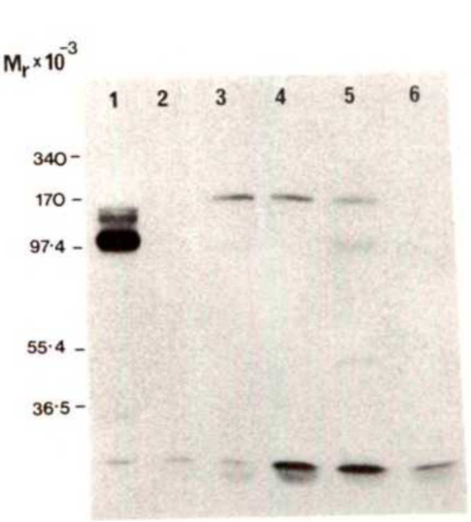

Fig 3. Immunoprecipitation of LFA-1–like molecules on platelets. Platelets surface labeled with ^{125}I were lysed with detergent and immunoprecipitated with: lane 1, APS; lane 2, M1/70; lane 3, I21/7.7; lane 4, ATS; lane 5, AMS; lane 6, control rabbit serum. Immunoprecipitates were then solubilized and electrophoresed on 6% to 12.5% gradient polyacrylamide gels, dried, and autoradiographed.

Comparison of LFA-1 family molecules on platelets, macrophages, and thymocytes. Figure 4 compares the mol wts of LFA-1–like molecules immunoprecipitated from platelets, macrophages, and thymocytes with I21/7.7, M1/70, AMS, and ATS. The α subunit immunoprecipitated from platelets and thymocytes with I21/7.7 showed a slightly higher mol wt (170,000) than the Mac-1 α subunit from macrophages (160,000) (Fig 4, lanes 3 to 5 and 7). The α subunit of the LFA-1 molecule immunoprecipitated by ATS from thymocytes is, however, not resolved (Fig 4, lane 2) because of the immunodominant T200 band.[29] LFA-1–like molecules immunoprecipitated from platelets with I21/7.7 did not show identity with the major platelet glycoproteins (Fig 4, lanes 1 and 4; Fig 3, lanes 1 and 3), although a minor platelet glycoprotein of about 170,000 mol wt (Fig 4, lane 1, arrowed) may be the α subunit of platelet LFA-1.

Glycosylation of platelet membrane proteins immunoprecipitated by LFA-1 family antibodies. Immunoprecipitation of solubilized platelets surface labeled with NaB^3H_4 produced similar results to those observed with iodination (Fig 5). Thus, I21/7.7 and ATS immunoprecipitated predominantly the α subunit of LFA-1, with trace amounts of

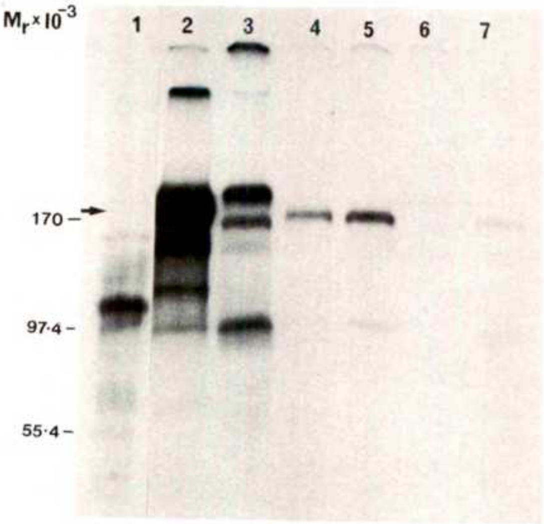

Fig 4. Immunoprecipitation of LFA-1 family molecules from platelets, thymocytes, and macrophages. Cells surface labeled with ^{125}I were lysed with detergent and immunoprecipitated as follows: lane 1, whole platelet lysate; lane 2, thymocytes with ATS; lane 3, macrophages with AMS; lane 4, platelets with I21/7.7; lane 5, thymocytes with I21/7.7; lane 6, macrophages with I21/7.7; lane 7, macrophages with M1/70. Immunoprecipitates were solubilized and electrophoresed on 6% to 12.5% gradient polyacrylamide gels, dried, and autoradiographed.

the β subunit being coprecipitated (lanes 2 and 3), whereas AMS precipitated both the α and β subunits (lane 4). M1/70 showed no evidence of specific immunoprecipitation products. A nonspecific band of 150,000 mol wt appeared in all immunoprecipitates except the serum control (lane 7) and M1/70 (lane 1), which was effectively a MoAb control. The platelet-specific MoAb IG3 and 7G6 immunoprecipitated a 210,000–mol wt glycoprotein from ^{3}H-labeled platelets (lanes 5 and 6), a band of similar mol wt also being observed in whole platelet lysates (lane 8). This band was not seen with iodinated platelet membranes (Fig 4, lane 1) or with immunoprecipitates of iodinated platelets (results not shown). Platelet LFA-1 did not correspond with any of the major cell surface components of ^{3}H-labeled platelets.

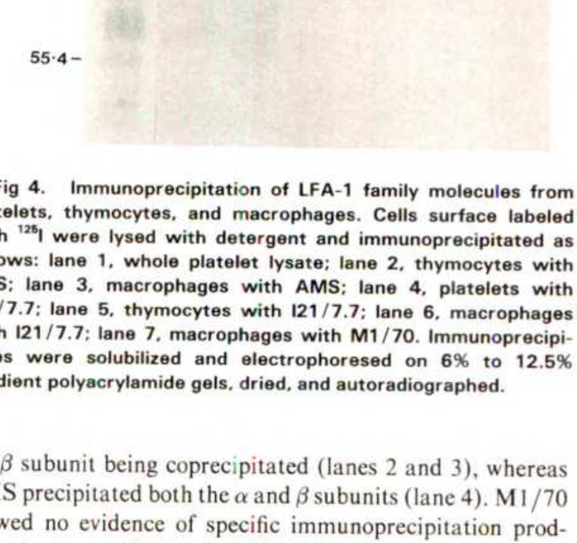

Fig 5. Glycosylation of LFA-1–like molecule on platelets. Platelets surface labeled with NaB^{3}H$_4$ were lysed with detergent and immunoprecipitated with: lane 1, M1/70; lane 2, I21/7.7; lane 3, ATS; lane 4, AMS; lane 5, IG3; lane 6, 7G6; lane 7, control rabbit serum; lane 8, whole platelet lysate. Immunoprecipitates were solubilized and electrophoresed on 6% to 12.5% gradient polyacrylamide gels, dried, and autoradiographed.

When M1/70 or AMS were used to immunoprecipitate Mac-1 from tritium-labeled macrophages (Fig 6A, lanes 1 and 2; Fig 6B, lane 1), a glycosylated protein of mol wt 210,000, was seen in addition to the 170,000–mol wt α subunit and 97,000–mol wt β subunit, the intensity of this band being similar to that of the β subunit of LFA-1. This 210,000–mol wt protein was not observed in immunoprecipitates of ^{125}I-labeled macrophages (Fig 4, lane 7).

Peptide mapping of platelet glycoproteins immunoprecipitated with I21/7.7. The β subunits of macrophage Mac-1, thymocyte LFA-1, and the LFA-1–like molecule from platelets showed similar peptide fragments following digestion with increasing quantities of V8-protease (Figs 7D to F). At the highest concentration of protease used, four fragments were produced, having mol wts of 49,000, 30,000, 19,000, and 13,000 daltons. Comparison of the peptide patterns of the α subunits immunoprecipitated by M1/70 from macrophages and I21/7.7 from thymocytes and platelets showed marked differences between M1/70 and I21/7.7 (Figs 7A to C). With high concentrations of protease the α subunit of Mac-1 produced five major peptides of mol wt 52,000, 36,000, 28,000, 17,000, and 5,000, whereas the α subunit of thymocyte LFA-1 and platelet LFA-1 produced seven fragments of mol wt 42,000, 27,000, 25,000, 19,000, 13,000, and two below 1,000 daltons.

Functional studies on platelet LFA-1. Two different functional assays were used in an attempt to ascribe functional significance to platelet LFA-1. The first series of experiments assessed the effect of LFA-1 MoAb on ADP-dependent platelet aggregation. Table 2 shows that although APS markedly inhibited platelet aggregation monoclonal antibodies against LFA-1 and Mac-1 had no significant effect. Likewise, in a second series of experiments in which platelet immune adherence was measured by an erythrocyte agglutination assay involving antibody and complement, no significant effect of antibodies against LFA-1 was observed (Fig 8). In contrast, M1/70 and APS showed extensive inhibition of erythrocyte agglutination.

Table 2. Effect of LFA-1 Family MoAb on Platelet Aggregation

Treatment	Transmission (%)*
Control NSI	48 ± 4
LFA-1 (I21/7.7)	49 ± 3
LFA-1 (M17/5.1.9)	46 ± 3
Mac-1 (M1/70)	44 ± 3
GP 210 (IG3)	39 ± 4
Control Serum 1:10	45 ± 1
APS/E 1:10	14 ± 4

*One hundred percent transmission was determined with PPP. MoAb and serum were diluted a further 1:4 in the reaction mixture. Results are the average of two to four experiments.

Abbreviation: GP, glycoprotein.

DISCUSSION

In this study the presence of an LFA-1–like heterodimer on mouse platelets has been demonstrated by several techniques. Using ELISA and indirect immunofluorescence assays, the LFA-1–specific MoAb I21/7.7[24] and M17/5.1.9[25] were shown to bind to mouse platelets. In contrast, M1/70 MoAb against Mac-1,[7] a cell surface molecule that belongs to the same family as LFA-1, showed no binding to mouse platelets by immunofluorescence and low binding by ELISA. Neither LFA-1 nor Mac-1 antibodies bound to human platelets (Table 1) despite the known cross-reactivity of M1/70 with human leukocytes.[30] Immunoprecipitation studies identified the cell surface molecule on platelets recognized by I21/7.7 as a dimeric molecule having subunit mol wts of 170,000 and 97,000. That this molecule is immunologically related to molecules of similar mol wt on macrophages is suggested by the ability of platelets to completely absorb antibodies in ATS that immunoprecipitate a 160,000/97,000 heterodimer from radiolabeled macrophages. Under these conditions, no loss of the 188,000–mol wt molecule identified by the T200 family MoAb I41/14.1 was observed (Fig 2). Preclearing experiments with M1/70 have shown that the 160,000/97,000–mol wt molecule on macrophages that is recognized by AMS and ATS is in fact Mac-1 (P.J. McCaffery, unpublished observations), suggesting that a platelet cell surface molecule is immunologically related to Mac-1. This conclusion is supported by the ability of ATS and AMS to immunoprecipitate a molecule indistinguishable from LFA-1 on platelets (Fig 3). However, in accordance with the low or negative antibody-binding data (Table 1, Fig 1), M1/70 produced no detectable immunoprecipitable products from radioiodinated platelets.

The mol wts of the cell surface molecules immunoprecipitated from platelets with I21/7.7 could not be distinguished from similar molecules immunoprecipitated from thymocytes (Fig 4), although the α subunit had a slightly higher mol wt than that of Mac-1 on macrophages. Peptide mapping showed identity of the β subunits immunoprecipitated by I21/7.7 from platelets and thymocytes and by M1/70 from macrophages, but distinguished the α subunit of Mac-1 from the α subunits immunoprecipitated by I21/7.7 from platelets and thymocytes. These results strongly support the contention that platelets express LFA-1 rather than a molecule immunologically related to LFA-1.

Platelet LFA-1 bears antigens that appear to be the major demonstrable determinants shared between platelets and thymocytes, ATS being essentially monospecific for platelet LFA-1 (Fig 3). In contrast to ATS, AMS immunoprecipitated a strong band at 97,000 mol wt that we have shown to include Pgp-1, a polymorphic glycoprotein of similar mol wt to the β subunit of LFA-1 and Mac-1 widely distributed on the hematopoietic cells.[31]

Platelet LFA-1 proteins are not major components of the platelet membrane (Fig 4, lane 1; Fig 5, lane 8) and are not immunoprecipitated by APS (Fig 3, lane 1), results that contrast with AMS and ATS, which immunoprecipitate Mac-1 and LFA-1 as major membrane proteins from the immunizing cell types (Fig 4, lanes 2 and 3). Thus, expression of LFA-1 on platelets appears to be markedly lower than on spleen cells (1.6×10^4 molecules/cell),[32] cytotoxic lymphocytes (10^5 molecules/cell),[25,32] or Mac-1 on thioglycollate-elicited macrophages (1.6×10^5 molecules/cell).[33] Possible identity of the α subunit of platelet LFA-1 needs to be resolved by two-dimensional electrophoresis or by immune clearance experiments with monoclonal antibodies. In this context it has been shown that human LFA-1 shows changes in mol wt following reduction that are similar to those of platelet glycoprotein Ia.[34]

Immunoprecipitation of tritium-labeled glycoproteins from macrophages with M1/70 consistently revealed a protein of mol wt 210,000 in addition to the α and β subunits of Mac-1 (Fig 6). This protein was not observed when macrophages were radioiodinated by the lactoperoxidase/glucose oxidase procedure, suggesting the absence of available tyrosine residues. Noncovalent association of this glycoprotein with Mac-1 has not been demonstrated in cross-linking studies that produce a complex with the predicted mol wt of the heterodimer,[35] and dimerization of the respective sub-

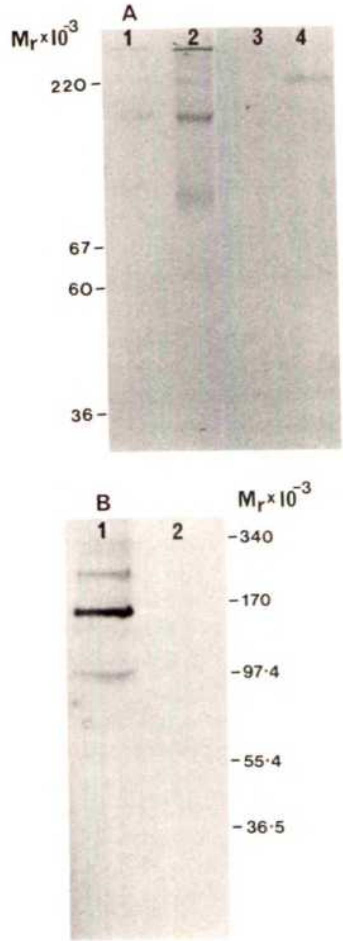

Fig 6. Glycosylation of cell surface molecules on macrophages recognized by M1/70. Macrophages surface labeled with NaB^3H_4 were lysed with detergent and immunoprecipitated with (A) lane 1, M1/70; lane 2, AMS; lane 3, control serum; lane 4, IG3 (gp 210); and (B) lane 1, M1/70; lane 2, control hybridoma supernatant (NS1).

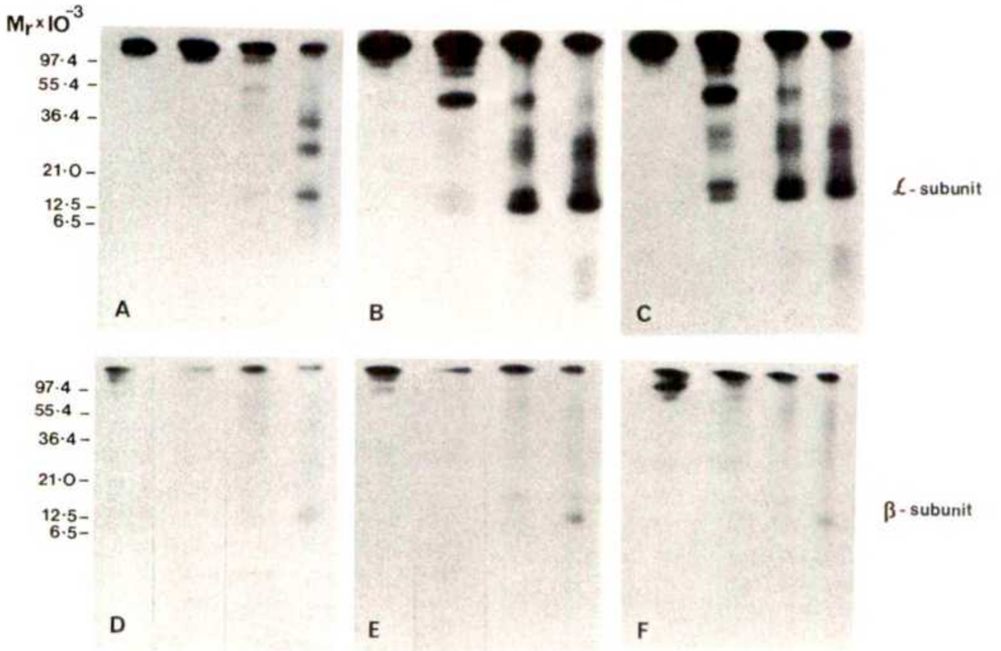

Fig 7. Peptide mapping of LFA-1 family molecules on platelets, thymocytes, and macrophages. The α and β subunits of LFA-1 family molecules isolated from platelets, thymocytes, and macrophages surface labeled with ^{125}I were digested with increasing amounts of V8-protease and analyzed on 15% polyacrylamide gels. α subunits isolated from (A) macrophages with M1/70; (B) thymocytes with I21/7.7; (C) platelets with I21/7.7. β subunits isolated from (D) macrophages with M1/70; (E) thymocytes with I21/7.7; (F) platelets with I21/7.7.

units is excluded by failure to demonstrate these glycoproteins in iodinated preparations. Thus, this element appears to be a monomeric glycoprotein not associated with the Mac-1/LFA-1 family but expressing epitopes recognized by M1/70.

Although Mac-1 could not be demonstrated on platelets by immunofluorescence or immunoprecipitation of radiolabeled platelets, its presence was clearly demonstrated by ELISA and by the ability of M1/70 to inhibit immune adherence of platelets to antibody-coated erythrocytes in the presence of complement (Fig 8). This evidence supports a large body of literature that implicates the presence of complement receptors on murine platelets.[36] However, it is possible that other complement receptors (eg, CR1) also contribute to platelet immune adherence.

Platelet function includes hemostasis,[37] immune adherence,[36] and cytolytic mechanisms.[38,39] It was therefore of considerable interest to determine whether platelet LFA-1 plays a role in platelet binding to target structures, in a manner analogous to the way in which LFA-1 is involved in the magnesium-dependent adherence of cytotoxic lymphocytes, NK cells, T-helper cells, and polymorphonuclear leukocytes to target structures.[11-13,40,41] Initial studies with I21/7.7 have shown neither inhibition of the ADP-dependent aggregation (Table 2) nor inhibition of the platelet-dependent agglutination of antibody-coated erythrocytes in the presence of complement (Fig 8). In contrast, I21/7.7 is known to inhibit cytolysis by cells from mixed lymphocyte cultures and by cytotoxic T-cell lines.[40] It is thus possible that a functional role for platelet LFA-1 may be able to be

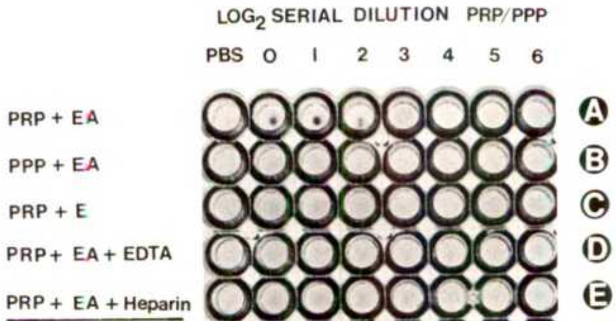

Fig 8. Effect of LFA-1 family MoAb on platelet immune adherence. In rows A to E, PRP or PPP was serially diluted (final volume, 25 μL) and EDTA (final concentration, 10 mmol/L) on heparin (final concentration, 40 units/mL) added prior to the addition of erythrocytes or EA. In rows F to J, antibody was serially diluted (12.5 μL) prior to the addition of PRP (12.5 μL) and EA (25 μL).

demonstrated in platelet-dependent cytotoxicity,[38,39] platelet adhesion to tumor cells or involvement in tumor metastasis,[42,43] or platelet aggregation stimulated by agents other than ADP, eg, collagen, ristocetin, or thrombin.

Recently the *N*-terminal amino acids of LFA-1 and Mac-1 have been shown to exhibit significant sequence homology with leukocyte interferon.[44] These results suggest that cell-cell signaling mechanisms involving interferonlike sequences on cell surfaces may have evolved in parallel with those involving interferon, and when considered in the context of present results, lead to the intriguing possibility that platelet LFA-1 may interact with the interferon receptor or a similar receptor molecule on target cells.

The demonstration of LFA-1 on the cell surface of mouse platelets extends the range of cell types expressing this protein and further establishes this family of proteins as a widespread group of antigenically dominant functional determinants on the surface of hematopoietic cells similar to the widespread occurrence of T200 family molecules.[29,45] The functional significance of LFA-1 on mouse platelets remains to be determined.

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REFERENCES

1. Phillips DR, Agin PP: Platelet plasma membrane glycoproteins: Evidence for the presence of nonequivalent disulfide bonds using nonreduced-reduced two dimensional gel electrophoresis. J Biol Chem 252:2121, 1977

2. McGregor JL, Clementson KJ, James E, Luscher EF, Dechavanne M: Characterization of human blood platelet membrane proteins and glycoproteins by their isoelectric point (pI) and apparent molecular weight using two-dimensional electrophoresis and surface-labelling techniques. Biochim Biophys Acta 599:473, 1980

3. Jenkins CSP, Phillips DR, Clementson KJ, Meyer D, Larrieu M-J, Luscher EF: Platelet membrane glycoproteins implicated in ristocetin-induced aggregation: Studies of the proteins on platelets from patients with Bernard-Soulier syndrome and von Willebrand's disease. J Clin Invest 57:112, 1976

4. Phillips DR, Agin PP: Platelet membrane defects in Glanzmann's thrombasthenia: Evidence for decreased amounts of two major glycoproteins. J Clin Invest 60:535, 1977

5. Coller BS: Report of the working party on hybridoma-derived monoclonal antibodies to platelets. Thromb Haemost 51:169, 1984

6. Kurzinger K, Reynolds T, Germain RN, Davignon D, Martz E, Springer TA: A novel lymphocyte function associated antigen (LFA-1) cellular distribution, quantitative expression and structure. J Immunol 127:596, 1981

7. Springer T, Galfre G, Secher DS, Milstein C: Mac-1: A macrophage differentiation antigen identified by monoclonal antibody. Eur J Immunol 9:301, 1979

8. Sanchez-Madrid F, Simon P, Thompson S, Springer TA: Mapping of antigenic and functional epitopes on the α and β-subunits of two related mouse glycoproteins involved in cell interactions LFA-1 and Mac-1. J Exp Med 158:586, 1983

9. Sanchez-Madrid F, Nagy FA, Robbins E, Simon P, Springer TA: A human leukocyte differentiation antigen family with distinct α-subunits and a common β-subunit: The lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac1), and the p150,95 molecule. J Exp Med 158:1785, 1983

10. Beller DI, Springer TA, Schreiber RD: AntiMac-1 selectively inhibits the mouse and human type three complement receptor. J Exp Med 156:1000, 1982

11. Davignon D, Martz E, Reynolds T, Kurzinger K, Springer TA: Monoclonal antibody to a novel lymphocyte function associated antigen (LFA-1): Mechanism of blockade of T lymphocyte-mediated killing and effects on other T and B lymphocyte functions. J Immunol 127:590, 1981

12. Dana N, Todd III RF, Pitt J, Springer TA, Arnaout MA: Deficiency of a surface membrane glycoprotein (Mol) in man. J Clin Invest 73:153, 1984

13. Kohl S, Springer TA, Schmalstieg FC, Loo LS, Anderson DC: Defective natural killer cytotoxicity and polymorphonuclear leukocyte antibody-dependent cellular cytotoxicity in patients with LFA-1/OKM-1 deficiency. J Immunol 133:2972, 1984

14. Berridge MV, O'Kech N: Surface antigens of murine hemopoietic stem cells. 1. Cross reactivity of antisera against differentiated hemopoietic cells with bone marrow stem cells. Exp Haematol 7:452, 1979

15. Ralph SJ, McCaffery P, Tan AS, Berridge MV: Binding of monoclonal antibodies that inhibit spleen colony formation to leukemic cell lines. Cancer Res 44:3825, 1984

16. Trowbridge IS, Ralph P, Bevan MJ: Differences in the cell surface proteins of mouse B and T cells. Proc Natl Acad Sci USA 72:157, 1975

17. Hubbard AL, Cohn ZA: The enzymatic iodination of the red cell membrane. J Cell Biol 55:390, 1972

18. Gahmberg CG, Andersson LC: Selective radioactive labelling of cell surface sialoglycoproteins by periodate-tritiated borohydride. J Biol Chem 252:5888, 1977

19. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680, 1970

20. Laskey RA, Mills AD: Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Lett 82:314, 1977

21. Skinner MK, Griswold MD: Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole in acetic acid and its comparison with existing procedures. Biochem J 209:281, 1983

22. Laskey RA, Mills AD: Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56:335, 1975

23. Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK: Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252:1102, 1977

24. Trowbridge IS, Omary MB: Molecular complexity of leukocyte surface glycoproteins related to the macrophage differentiation antigen Mac-1. J Exp Med 154:1517, 1981

25. Ware CF, Sanchez-Madrid F, Krensky AM, Burakoff SJ, Strominger JL, Springer TA: Human lymphocyte function associated antigen (LFA-1): Identification of multiple antigenic epitopes and their relationship to CTL-mediated cytotoxicity. J Immunol 131:1182, 1983

26. Vilella R, Lozano T, Mila J, Borche L, Ercilla G, Ordinas T, Vives J: An antiplatelet monoclonal antibody that inhibits ADP and epinephrine-induced aggregation. Thromb Haemost 51:93, 1984

27. Berridge MV, Ralph SJ, Tan AS: Cell-lineage antigens of the

stem cell-megakaryocyte-platelet lineage are associated with the platelet IIb/IIIa glycoprotein complex. Blood 66:76, 1985

28. Tung J-S, Scheid MP, Pierotti MA, Hammerling U, Boyse EA: Structural features and selective expression of three Ly-5$^+$ cell surface molecules. Immunogenetics 14:101, 1981

29. Ralph SJ, Berridge MV: Expression of antigens of the 'T200' family of glycoproteins on hemopoietic stem cells: Evidence that thymocyte cell lineage antigens are represented on 'T200'. J Immunol 132:2510, 1984

30. Ault KA, Springer TA: Cross reaction of rat anti-mouse phagocyte-specific monoclonal antibody (anti–Mac-1) with human monocytes and natural killer cells. J Immunol 126:359, 1981

31. Trowbridge IS, Lesley J, Schulte R, Hyman R, Trotter J: Biochemical characterization and cellular distribution of a polymorphic, murine cell surface glycoprotein expressed on lymphoid tissues. Immunogenetics 15:299, 1982

32. Kurzinger K, Reynolds T, Germain RN, Davignon D, Martz E, Springer TA: A novel lymphocyte function–associated antigen (LFA-1): Cellular distribution, quantitative expression, and structure. J Immunol 127:596, 1981

33. Springer TA, Ho M-K: Macrophage differentiation antigens: Markers for macrophage subpopulations and tissue localization, in Mitchell MS, Oettgen HF (eds): Hybridomas in Cancer Diagnosis and Treatment. New York, Raven, 1982, p 53

34. Sanchez-Madrid F, Krensky AM, Ware CF, Robbins E, Strominger JL, Burakoff ST, Springer TA: Three distinct antigens associated with human T-lymphocyte–mediated cytolysis: LFA-1, LFA-2 and LFA-3. Proc Natl Acad Sci USA 79:7489, 1982

35. Kurzinger K, Ho M-K, Springer TA: Structural homology of a macrophage differentiation antigen and an antigen involved in T-cell–mediated killing. Nature 296:668, 1982

36. Nelson DS: Immune adherence. Adv Immunol 3:131, 1963

37. Sixma JJ: The haemostatic plug, in Poller L (ed): Recent Advances in Coagulation. New York, Churchill Livingstone, 1981

38. Soper WD, Bartlett SP, Winn HJ: Lysis of antibody-coated cells by platelets. J Exp Med 156:1210, 1982

39. Joseph M, Auriault C, Capron A, Vorng H, Viens P: A new function for platelets: IgE-dependent killing of schistosomes. Nature 303:810, 1983

40. Sarmiento M, Loken MR, Trowbridge IS, Coffman RL, Fitch FW: High molecular weight lymphocyte surface proteins are structurally related and are expressed on different cell populations at different times during lymphocyte maturation and differentiation. J Immunol 128:1676, 1982

41. Beatty PG, Ledbetter JA, Martin PJ, Price TH, Hansen JA: Definition of a common leukocyte cell-surface antigen (Lp 95-150) associated with diverse cell-mediated immune functions. J Immunol 131:2913, 1983

42. Gasic GJ, Gasic TB, Galanti TB, Johnson T, Murphy S: Platelet–tumour cell interactions in mice. The role of platelets in the spread of malignant disease. Int J Cancer 11:704, 1973

43. Pearlstein E, Ambrogio C, Karpatkin S: Effect of antiplatelet antibody on the development of pulmonary metastases following injection of CT26 colon adenocarcinoma, Lewis lung carcinoma and B16 amelanotic melanoma tumour cells into mice. Cancer Res 44:3884, 1984

44. Springer TA, Teplow DB, Dreyer WJ: Sequence homology of the LFA-1 and Mac-1 leukocyte adhesion glycoproteins and unexpected relation to leukocyte interferon. Nature 314:540, 1985

45. Tung J-S, Scheid MP, Palladino MA: Different forms of Ly-5 within the T-cell lineage. Immunogenetics 17:649, 1983

Prognostic Implications of Morphology and Karyotype in Primary Myelodysplastic Syndromes

By Renee H. Jacobs, Michael A. Cornbleet, James W. Vardiman, Richard A. Larson,
Michelle M. Le Beau, and Janet D. Rowley

Forty-nine patients with primary myelodysplastic syndromes (MDS) were subclassified according to French-American-British (FAB) Cooperative Group criteria. Eight patients had acquired idiopathic sideroblastic anemia (AISA), ten had chronic myelomonocytic leukemia (CMMoL), 14 had refractory anemia (RA), nine had refractory anemia with excess blasts (RAEB), and five had refractory anemia with excess blasts in transformation (RAEB-T); three patients could not be subclassified. The actuarial median survival for patients with AISA or with RA had not been reached at 60 months of follow-up. The median survival times for patients with CMMoL, RAEB, and RAEB-T were 25, 21, and 16 months, respectively. The percentages of patients with each subtype who developed ANLL were none in AISA, 20% in CMMoL, 7% in RA, 56% in RAEB, and 40% in RAEB-T. Patients with CMMoL had a poor prognosis independent of transformation to acute nonlymphocytic leukemia (ANLL), whereas patients with RAEB and RAEB-T had a high incidence of transformation and short survival times. Clonal chromosomal abnormalities were present in bone marrow cells from 19 patients at the time of diagnosis, and two others developed an abnormal karyotype at the time of leukemic transformation. The most frequent abnormalities, including initial and evolutionary changes, were trisomy 8 (9 patients), deletion of 5q (4 patients), and deletion of 20q (4 patients). The median survival times were 32 months for patients with an abnormal karyotype, and 48 months for those with a normal karyotype ($P = 0.2$). Specific chromosomal abnormalities were not associated with particular histologic subtypes; however, a high percentage of patients with RAEB and RAEB-T had an abnormal clone (89% and 80%, respectively). The percentages of patients with clonal abnormalities were 13% for AISA, 20% for CMMoL, and 29% for RA. The MDS transformed to ANLL in 42% of patients with an abnormal karyotype, compared to 10% of those with an initially normal karyotype ($P < .01$). Among patients with RA, RAEB, and RAEB-T, the risk of leukemic transformation was confined to those with an abnormal karyotype ($P < .01$). Thus, in the present study, morphology and karyotype combined were the best indicators of outcome in patients with MDS.

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THE primary myelodysplastic syndromes (MDS) are a heterogeneous group of bone marrow disorders characterized by ineffective and dysplastic hematopoiesis in one or more cell lines. The most prominent manifestations are cytopenias related to progressive bone marrow failure. Evolution to acute nonlymphocytic leukemia (ANLL) occurs in 20% to 75% of cases.[1-5] The morphologic subclassification of these disorders remains controversial; however, the criteria proposed by the French-American-British (FAB) Cooperative Group are widely used.[6]

Clonal chromosomal abnormalities in bone marrow cells are recognized in ANLL de novo as well as in therapy-related MDS and ANLL. Moreover, certain abnormalities have significant clinicopathologic correlations.[7-9] With the use of current techniques, nonrandom cytogenetic abnormalities are observed in more than 80% of patients with ANLL de novo. In contrast, only 40% to 60% of patients with a primary MDS, or preleukemia, have clonal abnormalities. The usefulness of these clonal abnormalities for prediction of the natural course of the disease is uncertain.[10,11] At the Second International Workshop on Chromosomes in Leukemia, cytogenetic data for 244 patients with preleukemia were reviewed. Of 125 patients with an abnormal clone, 27% had evolved to ANLL, compared to 15% of patients with a normal karyotype. Trisomy 8 and loss of all or part of the long arm (q) of chromosomes 5 or 7 were the most common abnormalities noted. None of the specific chromosomal rearrangements such as the t(15;17) in acute promyelocytic leukemia, the t(8;21) in acute myelocytic leukemia with maturation, or the inv(16) in acute myelomonocytic leukemia with abnormal eosinophils, were present in these patients.

We now report in detail on the clinical, histopathologic, and cytogenetic data for 49 patients with primary MDS, expanding on our earlier series of eight patients.[12] We used these data for the following: (1) to determine whether specific cytogenetic abnormalities in MDS were correlated with the morphologic subtypes defined by the FAB criteria; (2) to evaluate whether the presence of cytogenetic abnormalities at diagnosis was correlated with the course of the disease or with the risk of transformation to ANLL; (3) to determine whether the risk of transformation to ANLL was related to the FAB subtype of MDS; and (4) to determine if the severity of dysplasia or the number of abnormal cell lines in the initial bone marrow specimen was correlated with cytogenetic abnormalities or with transformation to ANLL.

MATERIALS AND METHODS

The 49 patients described here include all those seen at the University of Chicago Medical Center between 1972 and 1984 with a diagnosis of a primary MDS that could be confirmed by morphologic study of bone marrow specimens. Clinical data were collected

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by review of each medical record. Patients were followed until death or through April 1985.

Cytogenetic analyses were performed as described previously.[8,13] Initial cytogenetic specimens were obtained before any cytotoxic therapy was given. Chromosome abnormalities were described according to The International System for Human Cytogenetic Nomenclature.[14] The criteria proposed by Rowley and Potter were used for the identification of abnormal clones.[13]

Differential counts of at least 100 white cells were performed on the peripheral blood samples, and of at least 400 nucleated cells on the marrow aspirate smears of each patient. The percentage of ringed sideroblasts was determined from smears or sections stained with a Prussian blue reaction for iron. Dyspoiesis was graded in each cell line as either absent, present, or present and severe. The designation of severe dysplasia was used when more then 50% of the cells in a given series demonstrated dysplastic features, according to the criteria of Juneja et al.[15] Abnormal localization of immature precursors (ALIP) was determined according to the criteria of Tricot et al,[16] except that we used paraffin-embedded, 4 to 5 μm thick sections of marrow rather than plastic-embedded marrow specimens. The criteria of the FAB group were used for the diagnosis and classification of each patient.[6] If the FAB criteria could not be applied, cases were considered unclassifiable. Acute leukemia was diagnosed when more than 30% type I and type II blasts were present in the marrow.[17]

Survival was calculated from the time of diagnosis of MDS. Analyses of categorical data were performed with the standard Pearson r × c chi-square statistic. Actuarial survival curves were constructed according to the methods of Berkson and Gage and their significance was tested with a modification of the Wilcoxon method.[18,19]

RESULTS

Clinical characteristics. The clinical characteristics of the 49 patients with primary MDS are summarized in Table 1. There were eight patients with acquired idiopathic sideroblastic anemia (AISA), 10 with chronic myelomonocytic leukemia (CMMoL), 14 with refractory anemia (RA), nine with refractory anemia with excess blasts (RAEB), and five with refractory anemia with excess blasts in transformation (RAEB-T). All patients with RAEB-T had 20% to 30% blasts in the bone marrow. We were unable to classify three patients according to FAB criteria. In one of these cases, there was severe dysgranulopoiesis and no increase in the number of blasts. In the other two, despite severe trilineage dysplasia, blasts were also not increased in number. Thus, the diagnosis of RA would be inappropriate because the FAB guidelines state that the granulocytic and megakaryocytic series almost always appear normal in that particular disorder.

The patients included 28 males and 21 females, ranging in age from 3 to 85 years; the median age for the entire group was 62 years. Patients with RA were significantly younger than those with the other subtypes of MDS.

The hematologic features at diagnosis are also summarized in Table 1. There was wide variation in the initial hemoglobin concentrations, leukocyte counts, and platelet counts among these patients. The only notable difference in these parameters among the FAB subtypes of MDS was the absence of thrombocytopenia and leukopenia in patients with AISA. Anemia, with its associated symptoms, was the most common hematologic abnormality and was observed in all subtypes of MDS. Only seven patients (14%) had initial hemoglobin values greater than 12 g/dL. At diagnosis, the absolute neutrophil count was less than 1000/μL in only four patients. This was an incidental finding in two patients presenting with anemia. The third had a fever of unknown origin. The fourth patient, who was 3 years old, had an immunodeficiency syndrome characterized by recurrent perirectal abscesses and skin infections. At diagnosis, five patients had life-threatening thrombocytopenia (less than 20,000 platelets/μL). Four of these patients reported easy bruising, but none had significant bleeding.

No patient had palpable lymphadenopathy. A small percentage of patients within each subtype, except AISA, had splenomegaly. However, the degree of splenomegaly was mild, with the spleen ranging in size from being just palpable to extending three centimeters below the costal margin. Hepatomegaly was noted only in three patients with CMMoL, and each of these patients also had splenomegaly.

Table 1. Patient Characteristics at Diagnosis of Myelodysplastic Syndromes

	AISA	CMMoL	RA	RAEB	RAEB-T	Unclassifiable
Number of patients	8	10	14	9	5	3
Median age (yr)	68	71	49	68	62	54
(range)	(41–79)	(55–85)	(23–73)	(55–76)	(29–66)	(3–73)
Median Hgb (g/dL)	9.2	9.3	8.3	9.8	9.9	9.6
(range)	(6.2–10.7)	(7.5–13.5)	(3.1–13.0)	(6.4–11.8)	(8.0–10.5)	(8.5–10.6)
Median WBC count ($\times 10^{-3}/\mu L$)	4.8	31.4	6.5	4.8	7.4	4.0
(range)	(2.3–6.6)	(9.3–71.0)	(2.5–13.8)	(1.1–7.8)	(1.1–84.0)	(3.2–4.8)
Median platelets ($\times 10^{-3}/\mu L$)	312	188	263	50	128	89
(range)	(250–570)	(12–613)	(3–503)	(10–193)	(15–212)	(15–163)
Splenomegaly only (%)	0	20	7	11	40	0
Hepatosplenomegaly (%)	0	30	0	0	0	0
Skin infiltration (%)	0	10	0	0	0	0
Median survival (mo)	103+	25	120+	21	16	23
Percent with abnormal karyotypes	13	20	29	89	80	0
Percent transforming to ANLL	0	20	7	56	40	33

AISA, acquired idiopathic sideroblastic anemia; CMMoL, chronic myelomonocytic leukemia; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RAEB-T, refractory anemia with excess blasts in transformation; ANLL, acute nonlymphocytic leukemia; Hgb, hemoglobin; WBC, white blood cells.

Skin infiltration by leukemia cells was also seen in one patient with CMMoL.

Although none of these patients had previously received chemotherapy or radiotherapy, one patient within each FAB subtype had a history of exposure to potentially mutagenic agents in the environment. One patient with AISA reported 18 years of exposure to industrial chemicals. Patient No. 21 with CMMoL was a sheet metal worker who eventually developed acute myelomonocytic leukemia. Both were cytogenetically normal at diagnosis, but the latter patient demonstrated a clonal abnormality at the time of transformation to ANLL. Patient No. 3 with RA was employed for several years in a battery factory and, at diagnosis, had a del(5q); this patient's condition has remained stable for more than 31 months. Patient No. 11 with RAEB was employed in a paint factory and in asphalt road construction. His initial marrow specimen study revealed monosomy 7; 40 months after diagnosis, he developed acute myeloblastic leukemia with maturation (M2). Patient No. 18, with RAEB-T, had been an industrial hygiene inspector and had a del(7)(q22q36). His disease transformed to ANLL four months after diagnosis and he remains alive with disease.

No consistent treatment approach was taken with these patients because they were seen over a 12-year period. Methods of treatment varied within the FAB subtypes of MDS. In general, patients received platelet and red blood cell transfusions whenever necessary. Other therapeutic agents used for some patients included folic acid, vitamin B_{12}, pyridoxine, steroids, and subcutaneous cytarabine. Among the 11 patients who had transformation to ANLL, only one (patient no. 19) received cytotoxic therapy (subcutaneous cytarabine) prior to transformation. Three patients (nos. 8, 9, and 17) received high-dose cytarabine (2 to 3 g/m^2) as primary treatment for their MDS, after other measures to correct severe thrombocytopenia had failed. Patient no. 8 subsequently died of gastrointestinal bleeding and sepsis while granulocytopenic. Patient no. 9 has had an unmaintained complete response for more than 11 months. Patient no. 17 had a complete hematologic and cytogenetic remission for one year but later relapsed and died of refractory leukemia.

The actuarial survival calculated for each FAB subtype of MDS is shown in Fig 1. For patients with AISA, the cumulative proportion surviving at 24 months was 80%, and the longest survivor was alive at 103 months. For patients with RA, the cumulative proportion surviving at 24 months was 89%, and the longest survivor was alive at 120 months. The median survival times for patients with CMMoL, RAEB, and RAEB-T were 25 months, 21 months, and 16 months, respectively. Patients with AISA and RA had median survivals that were significantly better than those for CMMoL, RAEB, and RAEB-T combined.

Of the 49 patients in this series, 19 had a clonal chromosomal abnormality at diagnosis. Of these, there were seven deaths due to ANLL, two deaths due to the primary MDS, and three deaths due to unrelated or unknown causes. In comparison, of the 30 patients with normal karyotypes, 14 have died; three deaths were due to ANLL, five to the primary MDS, and six to unrelated causes. The median survival for patients with a normal karyotype at diagnosis was 48 months, and for patients with an abnormal karyotype, 32 months. This difference was not statistically significant ($P = 0.2$). The actuarial survival curves for patients grouped according to normal and abnormal initial karyotypes are shown in Fig 2.

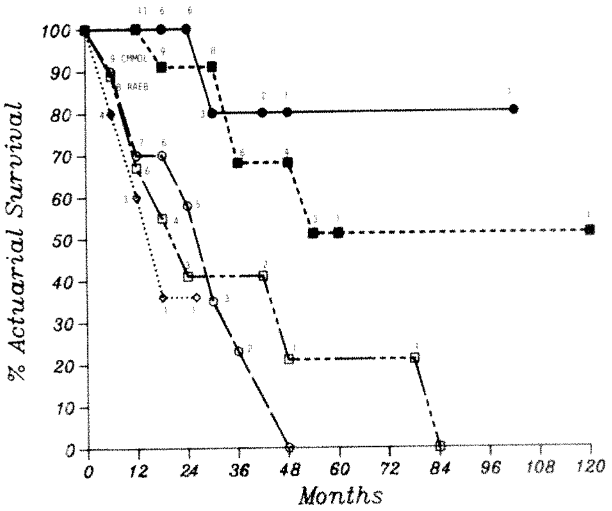

Fig 1. Actuarial survival in months according to FAB subtype. ●— AISA (n = 8); ○—— cmmol (n = 10); □ ——— RAEB (n = 9); ■--- RA (n = 13); ◇···· RAEB-T (n = 5). $P = 0.1$.

Transformation to ANLL was observed in 11 patients (22%), after a median interval of 8 months following the diagnosis of MDS (see Table 2). The median survival following transformation to ANLL was one month. Of the patients who developed ANLL, one initially had RA, two had CMMoL, five had RAEB, two had RAEB-T, and one was unclassifiable. The most frequent FAB subtypes of ANLL were myeloblastic leukemia with maturation (M2) in four patients and acute myelomonocytic leukemia (M4) in three patients; one patient had acute myeloblastic leukemia

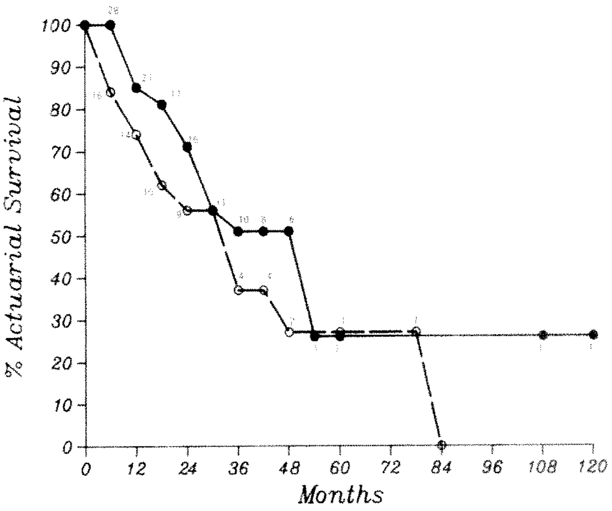

Fig 2. Actuarial survival according to the presence of a normal versus an abnormal karyotype at diagnosis. ●— Normal (n = 29); ○-- Abnormal (n = 19). $P = .2$.

Table 2. Cytogenetic Abnormalities in Primary MDS

MDS Subtype	Patient No.	Age/Sex	Survival After Diagnosis (Mos)	Survival After Transformation To ANLL (Mos)	FAB Subtype At Transformation To ANLL	Specimen*	Method†	Number of Metaphase Cells Examined	% BM Cells With Normal Karyotype	Karyotype of Abnormal Cells
Patients With Cytogenetic Abnormalities at Diagnosis										
AISA	1	41/M	26+			BM	D	16	0	46,XY,del(11)(q11q25)(88%)/47,same, +8(12%)
RA	2	28/F	25+			BM	24	25	17	46,XX,del(5)(q14q34)(83%)
	3	59/M	42+			BM	24, 48M	20	35	46,XY,del(5)(q15q33)(45%)/ 45,same, −20(20%)
	4	36/F	57+			BM	24, 24M	23	70	46,XX,del(13)(q11q14)(30%)
	5	49/F				BM	24	8	63	46,XX,t(9;22)(q34;q11)(37%)
			33	1	M1				Poor Sample	‡46,XX,t(9;22)
CMMoL	6	71/M	31			BM	D, 24	16	31	46,XY,del(13)(q11q14)(69%)
	7	85/M	3			BM	D	19	0	47,XY, +8(100%)
RAEB	8	62/M	16			BM	24, 48M	21	30	46,XY,del(20)(q11.2q13.3)(20%)/ 46,same,del(15)(q23q24)(50%)
	9	76/M	16+			BM	24, 48M	49	19	47,XY, +8(81%)
	10	75/F	79			BM	24	13	54	46,XX,del(20)(q11.2q13.3)(46%)
	11	55/M				BM	24	15	20	45,XY, −7(80%)
			42	2.5	M2	BM	48M	15	0	‡45,XY, −7,del(12)(p11p13)(80%)/ 44,same, −13(20%)
	12	61/F				BM	24	20	30	45,XX, −17,del(5)(q11q31),t(6;?)(p25;?), t(12;?)(p13;?)+DM(10%)/ 45,same,del(9)(q13q22)(35%)/ 46,same, +8(10%)/other related cells (15%)
			5	1	M2	BM	24	21	41	‡§45,XX, −17,del(5),6p+ ,12p+(14%)/ 45,same,del(1)(q21),t(3;?)(q11;?)(9%)/ 46,XX,del(6)(q21q26)(36%)
	13	68/F				BM	48M	24	0	46,XX,del(20)(q11.2q13.3)(25%)/ 47,same, +8(75%)
			21	2	M2	BM, PB	24(PB), 48M(BM)	22	9	‡46,XX,del(20)(q11.2q13.3)(73%)/ 47,same, +8(18%)
	14	75/M				BM	48M	15	73	§46,XY,t(1;2)(q12;q37)(20%)/ 46,XY,t(3;12;12)(q23;p13;q15)(7%)
			9	1	M2	BM, PB	24(PB); D, 24(BM)	35	30	‡§46,XY,t(1;2)(21%)/46,XY,t(3;12;12)(9%)/ 45,XY, −19,del(5)(q15q33),der(5), t(5;19)(q33;p13),dic(12;19)(p11;p13) (30%)/others(10%)
	15	68/M				BM	D, 24	15	93	47,XY, +8(7%)
						BM	D, 24	27	67	47,XY, +8(33%)
			8	0.1	M4	BM	D, 24	37	100	‡46,XY
RAEB-T	16	62/F	3			BM	24	11	0	47,XX, +8(100%)
	17	66/F	16			BM	24	16	0	47,XX, +8(100%)
	18	29/M				BM	24,48,48M	20	5	46,XY,inv(3)(q21q26),del(7)(q22q36)(70%)/ 46,XY,inv(3),ins(7;?)(?p13;?)(25%)
			27+	23+	Unclassifiable	PB	24	12	0	‡46,XY,inv(3)(q21q26),del(7)(q22q36)(100%)
	19	46/F				BM	D,24,48M	18	83	46,XX,t(9;?)(p24;?)(17%)
			15+	4+	M4	BM	24	30	66	‡46,XX,t(9;?)(p24;?)(27%)/ 46,XX,t(6;17)(p23;q21)(7%)
Cytogenetic Abnormalities in Patients With Initially Normal Karyotypes Who Transformed to ANLL										
CMMoL	20	55/M	6	2	M4	BM	D,24	12	0	‡48,XY, +11, +11(100%)
	21	57/F	24	Noted at Autopsy	Unclassifiable					Not Done
Unclassifiable	22	2½/F	22	0.5	M5a	BM	24	12	83	‡46,XX,t(9;?)(q21;?)(17%)

*BM, bone marrow; PB, peripheral blood

†D, direct preparations; 24, cells cultured for 24 hours; 24M and 48M, cells cultured in phytohemagglutinin-leukocyte conditioned medium and synchronized with methotrexate for 24 and 48 hours, respectively

‡Karyotype at transformation to ANLL

§Two or more independent clones

without maturation (M1), and one had poorly differentiated monoblastic leukemia (M5a). Two patients developed ANLL that could not be readily subclassified according to FAB criteria.

Cytogenetic analyses. For the 19 patients who had an abnormal karyotype at the time of diagnosis of MDS, details of the cytogenetic abnormalities are shown in Table 2. The most frequent abnormalities were trisomy 8 in five patients, del(5q) in three patients, and del(20q) in three patients. Each of these was also noted as an evolutionary change, with trisomy 8 occurring most often. These abnormalities were occasionally found together in the same patient, usually as a result of evolution of the initial abnormal karyotype.

All deletions of 5q were interstitial although they were thought to affect somewhat different bands; the proximal breakpoints occurred in bands 5q11 to 5q15, and the distal breaks occurred in bands 5q31 to 5q34. All three patients with del(20q) had similar abnormalities with an interstitial deletion involving bands 20q11.2 to 20q13.3. The other cytogenetic abnormalities were variable; however, two patients (No. 4 and No. 6) had exactly the same interstitial deletion of 13[del(13)(q11q14)]. One patient (No. 5) had a typical t(9;22) and has been reported on in detail.[20] Patient No. 12 with a del(5q) had a complex pattern of related chromosomally abnormal clones. When the first sample was obtained from this patient, the largest clone (45%) had a translocation of unidentified material to 6p(p23 or 25) as well as an interstitial deletion of 9q resulting in loss of bands q13q22 or q22q34. Although the patient did not have the typical t(6;9)(p23;q34) which may be associated with increased marrow basophilia,[21] a complex rearrangement resulting in the juxtaposition of the critical genes on 6p23 and 9q34 could have occurred. This interpretation was suggested by the fact that the marrow from this patient contained 25% basophils.

The association of a specific karyotypic abnormality with the MDS subtypes appeared variable. Thus, a +8 was seen in all groups except RA, and it was present in one-half of the patients with RAEB, either initially or after evolution. A del(5q) was observed in both RA and RAEB, whereas all three patients with the del(20q) had RAEB. Loss of No. 7 and del(7q) were noted in RAEB and RAEB-T, respectively. The other abnormalities were randomly distributed throughout the subgroups. Overall, a clonal abnormality was detected in one of the eight patients with AISA, in two of the ten with CMMoL, in four of the 14 with RA, in eight of the nine with RAEB, and in four of the five with RAEB-T.

Two of the 19 patients (Nos. 12 and 14) had two or more independent abnormal clones, either initially or at the time of transformation to ANLL. This was in marked contrast to the frequency of unrelated clones in our concurrent series of patients with ANLL de novo; that frequency was 1 in 196 patients ($P < .001$; unpublished results).[22]

Clinical correlations with cytogenetic abnormalities. Eight of the 19 (42%) patients with an initially abnormal karyotype developed ANLL, compared to three of the 30 (10%) patients with a normal karyotype. Thus, there was a significant association between the presence of an initial chromosome abnormality and the subsequent development of ANLL ($P < 0.01$). Among patients with RA, RAEB, or RAEB-T, the risk of ANLL was confined to those with an abnormal karyotype (8/16 *v* 0/12 with a normal karyotype; $P < .01$).

In this small series, it is difficult to establish whether patient survival from the time of diagnosis or the length of time to transformation to ANLL was related more closely to the chromosome pattern or to the FAB subtype of the MDS. There appears to be no correlation between the karyotype and specific subtypes of MDS, except possibly for del(20q) and RAEB. Of the five patients with trisomy 8, four have died, and the fifth developed ANLL; survival from the time of diagnosis was relatively short (3 to 16 months). In contrast, although all three patients with del(20q) have died (one after ANLL), their survival times ranged from 16 to 79 months. The two patients (Nos. 11 and 18) with abnormalities in chromosome No. 7 had marked differences (39.5 months and 4 months, respectively) in the length of the preleukemic phase of their disease before transformation to ANLL. Two patients (No. 2 and No. 3) with RA and del(5q) showed this aberration alone or as a mosaic of two abnormal clones; both patients are alive at 25 and 42 months, respectively. In contrast, two patients (No. 12 and No. 14) with RAEB had del(5q) as part of a complex karyotype, and their MDS transformed to ANLL. Patient No. 12 developed ANLL after 4 months, Patient No. 14 had two independent clones and developed a third independent clone with del(5q) at the time of transformation to ANLL, 8 months after the diagnosis of MDS.

Of the three patients (Nos. 20 to 22) with an initially normal karyotype who later developed ANLL, two had CMMoL. In one of these, acute leukemia was unexpectedly found at autopsy, and analysis of the karyotype was not repeated. When the second patient developed ANLL, 100% of his bone marrow cells had the karyotype 48,XY,+11,+11. The third patient had an unclassified type of MDS; the karyotype at transformation was 46,XX in 83% of cells and 46,XX,t(9;?)(q21;?) in 17%.

Morphologic features. Every patient in the study showed evidence of some dyspoiesis in at least one marrow cell line. Severe dysplasia, as described by Juneja et al,[15] was found in at least one cell line in 36 of the patients. Nine patients initially had severe dysplasia in two cell lines, and 10 additional patients had severe (trilineage) dysplasia in the granulocytic, erythroid, and megakaryocytic cell lines. These latter 10 patients were in the RAEB (2 patients), RAEB-T (3 patients), CMMoL (3 patients), and unclassified (2 patients) categories. Five of the 10 patients (50%) with severe trilineage dysplasia had transformation to ANLL, compared to 15% of those without severe trilineage dysplasia ($P < .05$). In addition, 10 of the 11 patients (91%) whose disease transformed to ANLL had severe dysplasia initially in at least two cell lines (seven in the granulocytic and megakaryocytic lines, and three in the erythroid and megakaryocytic lines). The median survival time of patients with trilineage dysplasia was 23 months, compared to 46 months for patients without such extensive dysplasia. The difference in survival times of patients, regardless of whether ANLL supervened, approached statistical significance based on the degree and extent of dyspoiesis ($P = .05$). There was no significant correlation between karyotypic abnormalities and the severity of dysplasia.

In this study, 28 of 43 patients (65%) from whom adequate bone core biopsy specimens were available demonstrated ALIP as defined by Tricot et al.[16] ALIP refers to clustering of myeloblasts and promyelocytes centrally in the bone marrow, rather than in the usual location along the endosteal surface. Every patient classified as having RAEB, RAEB-T, or CMMoL had this feature, as did three of the eight patients with AISA and one patient with RA. We could detect no significant associations between ALIP and abnormal karyotypes. Each of nine evaluable patients who had transformation to ANLL demonstrated ALIP; 19 other patients with ALIP have not developed leukemia. Although this difference was significant ($P < .05$), the presence or absence of ALIP appears to have no additional predictive value for either survival or transformation to ANLL over the FAB classification.

Atypical, hypolobulated megakaryocytes were present in patient no. 2, the single patient in this study who had a del(5q) as the sole chromosomal aberration. This patient, classified as RA, showed minimal dyserythropoiesis and dysgranulopoiesis. There has been no change in her disease over 4 years. Similar megakaryocytic abnormalities were present in two additional patients (nos. 3 and 12), both of whom had a del(5q) in combination with other chromosome changes. Both of these patients also had severe dyserythropoiesis at the time of diagnosis and one had an increased number of blasts.

DISCUSSION

We have described the clinical features, cytogenetic abnormalities, and morphologic findings in 49 patients with primary MDS. Correlations were sought among subsets of patients classified according to the morphologic criteria of the FAB group. Taken together, the cytogenetic and morphologic findings identified subsets of prognostic importance.

Our data indicate that particular chromosomal abnormalities are not associated with specific FAB subtypes, with the possible exception of del(20q) in RAEB. However, we found the presence of a cytogenetic abnormality at diagnosis to have prognostic significance. In our series, 39% of patients had a cytogenetic abnormality at diagnosis (median survival, 32 months); 42% of these developed ANLL compared to only 10% of those with a normal karyotype (median survival, 48 months). Comparable results have been reported by other investigators.[10,23,24] At the Second Workshop, patients with cytogenetic abnormalities had progressed to ANLL more often than had those with a normal karyotype (27% *v* 15%, $P < 0.05$).[10] Todd and Pierre also noted a significant difference in median survivals between patients with normal and abnormal karyotypes (3.4 *v* 1.85 years, respectively; $P < 0.004$).[24]

Two patients (nos. 12 and 14) had several independent abnormal clones; this is rarely observed in ANLL de novo. The unexpectedly high incidence of multiple independent clones in the present series may be an important feature of MDS, although its significance is unclear at present.

The most frequent cytogenetic abnormality seen in our series was trisomy 8 which is common both in ANLL de novo and in primary MDS at the time of transformation to ANLL.[9,10,25] The prognostic significance of other karyotypic abnormalities, such as del(5q), is unclear.[8,26] In the present study, the two patients who had a clone with only a 5q− had RA and apparently stable disease. On the other hand, both patients with a complex karyotype including a 5q− abnormality had RAEB that later transformed to ANLL. Whether the complex karyotype was "responsible" for a more dysplastic marrow with a greater likelihood of ANLL, or whether the degree of dysplasia was independent of the karyotype is uncertain.

An interstitial deletion of the long arm of chromosome 20 (breakpoints q11.2 and q13.3) was seen in three of our patients, all of whom had RAEB. Del(20q) is uncommon among patients with ANLL de novo or therapy-related ANLL but has been observed in some patients with polycythemia vera, AISA, and other myeloproliferative disorders.[27-29]

Two patients in our series had deletions involving chromosome 7, and both had histories of exposure to potentially mutagenic materials. By comparison, among 47 patients with therapy-related MDS evaluated at the University of Chicago, 45 (96%) had clonal abnormalities involving either chromosome 5 or chromosome 7.[30]

In our study, the prognostic value of the FAB classification system for MDS was confirmed, as it has been by others. None of our patients with AISA had transformation to ANLL. Other investigators have also reported that patients with AISA had a relatively benign course, with a median survival time of about ten years.[31] Seven to 25% of patients reported in other series, however, eventually developed ANLL.[15,31,32]

As defined by the FAB criteria, RA, RAEB, and RAEB-T differ in the number of blasts present in the peripheral blood and bone marrow, the presence or absence of Auer rods, and the lack of severe dysplasia in RA.[6] In our series, patients with RA were less likely to develop ANLL and had longer survival times than did patients with RAEB and RAEB-T. In two other studies, ANLL developed in 18% and 55% of patients with RAEB.[15,33] Thus, RAEB and RAEB-T are more often "preleukemic" disorders or the "forme fruste" of a rapidly progressive hematopoietic neoplasm.

CMMoL is a poorly defined disorder characterized by monocytosis, usually occurring in elderly patients. These patients appear to have a poor prognosis independent of cytogenetic abnormalities at diagnosis. In our series, neither of the patients who had chromosomal abnormalities at diagnosis developed ANLL, but two with normal karyotypes did have transformation to ANLL. Death was most frequently due to progressive bone marrow failure. Solal-Celignay et al have reported a median survival time of 475 days in 35 patients with CMMoL, 20% of whom developed ANLL.[34] In another series, 30% of all patients eventually developed ANLL.[35]

Several attempts have been made to define other morphologic parameters that provide prognostic information in MDS in addition to the FAB subtype.[4,36-38] In this series, additional prognostic information was obtained using a scoring system for the severity of dysplasia, similar to that used by Juneja et al.[15] Patients with severe dyspoiesis in two or three cell lines were much more likely to develop ANLL than those with less severe abnormalities; severe abnormalities in the megakaryocytes were especially ominous. This finding is in agreement with Varela et al, who also emphasized the prognostic import of dysmegakaryocytopoiesis and dysgranulocytopoiesis for leukemic progression.[39]

Like Tricot et al,[16] we found that ALIP was associated with shorter survival times and an increased incidence of ANLL, but we did not find an association between ALIP and cytogenetic abnormalities at diagnosis. In both studies, the majority of patients with ALIP had CMMoL, RAEB, or RAEB-T. However, unlike Tricot, we found a significant survival advantage for patients with RA and AISA (median survival not reached at 120 months) compared to those with CMMoL, RAEB, and RAEB-T (median survival, 22 months) ($P = .0004$). Thus, we conclude that ALIP is strongly associated with the FAB subtype, and in our series, offered no additional prognostic information.

Taken together, the morphologic findings and cytogenetic abnormalities at diagnosis can identify patients with primary MDS who have a high likelihood of developing ANLL and whose survival times are limited. In this group of patients with a poor prognosis, the early use of intensive chemotherapy to eradicate the malignant clone prior to the emergence of overt leukemia may be justifiable.[40] In contrast, the use of cytotoxic therapy in patients with features suggesting a good prognosis is unwarranted.

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REFERENCES

1. Linman JW, Saarni MI: The preleukemic syndrome. Semin Hematol 11:93, 1974

2. Bloomfield CD, Brunning RD: Acute leukemia as a terminal event in non-leukemic hematopoietic disorders. Semin Oncol 3:297, 1976

3. Linman JW, Bagby GC: The preleukemic syndrome (hemopoietic dysplasia). Cancer 42:854, 1978

4. Greenberg PL: The smoldering myeloid leukemic states: Clinical and biologic features. Blood 61:1035, 1983

5. Nowell P, Finan J: Chromosome studies in preleukemic states: IV. Myeloproliferative versus cytopenic disorders. Cancer 42:2254, 1978

6. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the myelodysplastic syndromes. Br J Haematol 51:189, 1982

7. Pierre RV: Preleukemic syndromes. Virchows Arch B Cell Path 29:29, 1978

8. Rowley JD, Golomb HM, Vardiman JW: Nonrandom chromosome abnormalities in acute leukemia and dysmyelopoietic syndromes in patients with previously treated malignant disease. Blood 58:759, 1981

9. Le Beau MM, Rowley JD: Recurring chromosomal abnormalities in leukemia and lymphoma. Cancer Surveys 3:371, 1984

10. Second International Workshop on Chromosomes in Leukemia (1979): Chromosomes in preleukemia. Cancer Genet Cytogenet 2:108, 1980

11. Nowell PC: Cytogenetics in preleukemia. Cancer Genet Cytogenet 5:265, 1982

12. Streuli RA, Testa JR, Vardiman JW, Mintz U, Golomb HM, Rowley JD: Dysmyelopoietic syndrome: Sequential clinical and cytogenetic studies. Blood 55:636, 1980

13. Rowley JD, Potter D: Chromosomal banding patterns in acute leukemia. Blood 47:705, 1976

14. An international system for human cytogenetic nomenclature (1978): Report of the Standing Committee on Human Cytogenetic Nomenclature. Cytogenet Cell Genet 21:309, 1978

15. Juneja SK, Imbert M, Jouault H, Scoazec JY, Sigaux F, Sultan C: Haematologic features of primary myelodysplastic syndromes (PMDS) at initial presentation: A study of 118 cases. J Clin Pathol 36:1129, 1983

16. Tricot G, DeWolf-Peeters C, Viletinck R, Verwilghen RL: Bone marrow histology in myelodysplastic syndromes: II. Prognostic value of abnormal localization of immature precursors in MDS. Br J Haematol 58:217, 1984

17. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C: Proposals for the classification of the acute leukemias. Br J Haematol 33:451, 1976

18. Berkson J, Gage R: Calculation of survival rates for cancer. Proc of the Mayo Clinic 25:270, 1950

19. Gehan EA: A generalized Wilcoxon test for comparing arbitrarily singly censored samples. Biometrika 52:203, 1965

20. Roth DG, Richman CM, Rowley JD: Chronic myelodysplastic syndrome (preleukemia) with a Philadelphia chromosome. Blood 56:262, 1980

21. Pearson MG, Vardiman JW, Le Beau MM, Rowley JD, Schwartz S, Kerman SL, Cohen MM, Fleischman EW, Prigogina EL: Increased numbers of marrow basophils may be associated with a t(6;9) in ANLL. Am J Hematol 18:393, 1985

22. Larson RA, Le Beau MM, Vardiman JW, Testa JR, Golomb HM, Rowley JD: The predictive value of initial cytogenetic studies in 148 adults with acute nonlymphocytic leukemia: A 12-year study (1970–1982). Cancer Genet Cytogenet 10:219, 1983

23. Kardon N, Schulman P, Degnan TJ, Dubman DR, Davis J, Vinciguerra V: Cytogenetic findings in the dysmyelopoietic syndrome. Cancer 50:2834, 1982

24. Todd WM, Pierre RV: Preleukaemia: A long-term prospective study of 326 patients. Scand J Haematol 36:114, 1986 (suppl 45)

25. Koeffler HP, Golde DW: Human preleukemia. Ann Intern Med 93:347, 1980

26. Watt JL, King DJ, Palmer JBD, Davidson RJL: The heterogeneity of the 5q- chromosome marker in refractory anemia. Cancer Genet Cytogen 8:113, 1983

27. Testa JR, Kinnealey A, Rowley JD, Golde DW, Potter D: Deletion of the long arm of chromosome 20 [del(20)(q11)] in myeloid disorders. Blood 52:868, 1978

28. Davis MP, Dewald GW, Pierre RV, Hoagland HC: Hematologic manifestations associated with deletions of the long arm of chromosome 20. Cancer Genet Cytogenet 12:63, 1984

29. Miller JB, Testa JR, Lindgren V, Rowley JD: The pattern and clinical significance of karyotypic abnormalities in patients with idiopathic and postpolycythemic myelofibrosis. Cancer 55:582, 1985

30. Le Beau MM, Albain KS, Larson RA, Vardiman JW, Davis EM, Blough RR, Golomb HM, Rowley JD: Clinical and cytogenetic correlations in 63 patients with therapy-related myelodysplastic syndromes and acute nonlymphocytic leukemia: Further evidence for characteristic abnormalities of chromosome Nos. 5 and 7. J Clin Oncol 4:325, 1986

31. Kushner JP, Lee GR, Wintrobe MM, Cartwright GE: Idiopathic refractory sideroblastic anemia. Clinical and laboratory investigation of 17 patients and review of the literature. Medicine 50:139, 1971

32. Bitran J, Golomb HM, Rowley JD: Idiopathic acquired

refractory sideroblastic anemia: Banded chromosome analysis in six patients. Acta Haemat 57:15, 1977

33. Ayraud N, Donzeau M, Raynaud S, Lambert JC: Cytogenetic study of 88 cases of refractory anemia. Cancer Genet Cytogenet 8:243, 1983

34. Solal-Celigny P, Desaint B, Herrara A, Chastang C, Amar M, Vrocians M, Brousse N, Mancilla F, Renoux M, Bernard JF, Bolvin P: Chronic myelomonocytic leukemia according to FAB classification: Analysis of 35 cases. Blood 63:634, 1984

35. Groupe Francois de Cytogenetique Hematologique: Cytogenetics of chronic myelomonocytic leukemia (CMML): A multicentre study of 120 patients. Cancer Genet Cytogenet (submitted)

36. Coiffier B, Adeleine P, Viala JJ, Bryon PA, Fiere D, Gentillhomme O, Vuvan H: Dysmyelopoietic syndromes: A search for prognostic factor in 193 patients. Cancer 52:83, 1983

37. Rosenthal DS, Moloney WC: Refractory dysmyelopoietic anemia and acute leukemia. Blood 63:314, 1984

38. Mufti GJ, Stevens JR, Oscier DG, Hamlin TJ, Machin D: Myelodysplastic syndromes: A scoring system with prognostic significance. Br J Haematol 59:425, 1985

39. Valera BL, Chuang C, Woll JE, Bennett JM: Modifications in the classification of primary myelodysplastic syndromes: The addition of a scoring system. Hematol Oncol 3:55, 1985

40. Larson RA: Management of myelodysplastic syndromes. Ann Intern Med 103:136, 1985

CONCISE REPORT

Thrombocytopenia in Venocclusive Disease After Bone Marrow Transplantation or Chemotherapy

By B. Rio, G. Andreu, A. Nicod, J.P. Arrago, F. Dutrillaux, M. Samama, and R. Zittoun

Hepatic venocclusive disease (VOD) is a frequent complication of bone marrow transplantation (BMT). Analysis of 13 cases observed during a 3-year period in our BMT center shows that VOD is associated with a constant peripheral thrombocytopenia and refractoriness to platelet transfusion. These signs appear in the very early stage of VOD, five to ten days before the classical signs, painful hepatomegaly and sudden weight gain. Analysis of platelet consumption, frequency of platelet transfusion and platelet recovery, and examination of known causes of peripheral thrombocytopenia (mainly allo- and autoimmunization, disseminated intravascular coagulation [DIC] and splenomegaly) lead to the conclusions that this association is not coincidental. The exact mechanism of platelet consumption in VOD is unknown.
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HEPATIC venocclusive disease (VOD), was originally described by Bras in 1954[1] in Jamaican children treated with herbal medicinal teas containing pyrrolizidine alkaloids. *Senecio, Crotalaria,* and *Heliotropum* plants were found to be responsible for micro-epidemics of VOD in the Caribbean, India, Egypt, Hong Kong, Afghanistan, Mexico, and Arizona.[2-5] Alcohol was also shown to be a causative factor of VOD.[6,7] VOD was later described following treatment by cytarabine, 6-thioguanine, and azathioprine.[8-11] Noteworthy is the high incidence of this disease after bone marrow transplantation. Twenty percent of the patients undergoing allogeneic bone marrow transplantation eventually develop VOD, with a 75% average mortality rate.[12-15] Most cases occurred after the usual conditioning regimen with cyclophosphamide and total body irradiation. Isolated cases following autologous bone marrow graft have also been reported in patients preparated with mitomycine, DTIC, and nitrosourea.[16-20]

Between July 1982 and April 1985 we observed 13 cases of VOD. In addition to the clinical features classically described, ie, sudden painful hepatomegaly with postsinusoidal intrahepatic portal hypertension, we noticed that these patients were deeply thrombocytopenic and developed an early refractoriness to platelet transfusion. This association does not seem purely coincidental and we present these observations.

MATERIALS AND METHODS

Thirteen patients were diagnosed as having VOD. Nine of the cases followed allogeneic bone marrow transplantation for acute myeloblastic (2 cases) and lymphoblastic (5 cases) leukemia, chronic myeloid leukemia (1 case), and aplastic anemia (1 case). In the latter, preventive treatment of graft-versus-host disease involved use of cyclosporine; in the other patients, the treatment regimen used was methotrexate at days 1, 3, 6, and 11, and then weekly. In two cases VOD followed autologous bone marrow transplantation for acute leukemia and T-lymphoblastic lymphoma. The last two cases were observed after chemotherapy for relapsing leukemia, one myeloblastic and one lymphoblastic. Clinical characteristics and conditioning regimen used for the bone marrow transplantation are summarized in Table 1.

The diagnosis of VOD was confirmed either by transjugular liver biopsy or by postmortem examination in 10 patients: at least two of these three signs—central or suslobular vein occlusion, without thrombosis; congestion of centrilobular sinusoids; centrilobular fibrosis—were found. In the three remaining cases the diagnosis was based on the criteria defined by Shulman: clinical association of a 1.5 kg weight gain in 24 hours and concomitant sudden development of a painful hepatomegaly within the month following transplantation[12] and by echography (Table 2).

The 9 patients with VOD following allogeneic bone marrow transplantation were compared with all the patients who underwent allogeneic transplantation in our institution. In the two groups we analyzed and compared the following factors:

(1) Platelet consumption: number of platelet units, either random or single-donor platelet concentrates transfused during the 3 months following transplantation.

(2) Frequency of platelet transfusion: analysis concerned the first, second, and third platelet tranfusion after bone marrow graft and was expressed as the number of days between those platelet transfusions.

(3) Platelet recovery was calculated in cases of transfused single-donor platelet concentrates as follows:

$$R = \frac{\text{observed increment} \times \text{blood volume}}{\text{number of platelets infused}} \times 100$$

Blood volume was evaluated according to Nadler.[21] In all cases platelet count after transfusion was performed 16 to 18 hours after the end of the transfusion.

Since February 1984 we have studied the recovery after single-donor platelet concentrate transfusion. Therefore 23 patients could be analyzed for platelet transfusion recovery: 7 with VOD and 16 without VOD. Patients were considered refractory when the recovery was less than 25%. The mean recovery value in non-HLA immunized patients without fever of more than 38 °C and splenomegaly is 56 ± 15% (extreme values 38 to 80). HLA alloimmunization was assessed by standard microlymphocytotoxicity test on a panel of 18 lymphocytes at least once a week. Coagulation tests included prothrombin time and assay of vitamin K dependent factors, partial thromboplastin time, fibrinogen, fibrinogen degradation products, and soluble complexes. Statistical analysis was performed using Student *t* test and the chi-square test.

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Table 1. Clinical Status of Patients With VOD

Name	Diagnosis	Status	Treatment	GVH	Cause of Death	Survival Duration Following Therapy
1. JOI	T-ALL	2nd CR	BCNU/VP16/AraC/CPM TBI/Allo BMT	0	VOD/CMV	79
2. MOS	T-ALL	1st CR	CPM/TBI/Allo BMT	2	—	670+
3. BRI	cALL	Relapse	VP16/AraC/CPM TBI/AlloBMT	2	VOD	50
4. DJE	cALL	3rd CR	VP16/AraC/CPM TBI/AlloBMT	0	VOD	22
5. AZI	ALL	1st CR	CPM/TBI/AlloBMT	0	VOD/IP	59
6. BAH	cALL	Relapse	PDN/VCR/Aspa	—	VOD/Leukemia	81
7. VAN	T-NHL	PR	Busulfan/CPM/AutoBMT	—	Lymphoma	172
8. EZZ	AML	1st CR	CPM/TBI/AlloBMT	0	VOD/IP	55
9. BOZ	AML	1st CR	CPM/TBI/AlloBMT	0	VOD	90
10. FAK	AML	2nd CR	CPM/TBI/AutoBMT	—	—	348+
11. BOU	AML	Relapse	HD/AraC/Amsa	—	VOD/leukemia	24
12. DUP	CML	CP	CPM/TBI/AlloBMT	0	VOD	21
13. ABO	AA		CPM/TAI/AlloBMT	0	VOD	17

Abbrev: ALL, acute lymphoblastic leukemia; T, type T; c, common; T-NHL, T-lymphoblastic lymphoma; AML, acute myeloblastic leukemia; AA, aplastic anemia; CP, chronic phase; CR, complete remission; PR, partial remission; TBI, total body irradiation; TAI, thoracoabdominal irradiation; BMT, bone marrow transplantation; VOD, veno-occlusive disease; CMV, cytomegalovirus; IP, interstitial pneumonia; GVH, graft-versus-host disease.

RESULTS

In all 13 observations there existed a thrombocytopenia, refractory to platelet transfusion. Amidst our allogeneic bone marrow transplant patients the number of platelet units required was significantly greater in those developing VOD ($P < 0.05$). Both groups received an average of 6 ± 2 units of single donor platelets; a mean of 37 pooled random donor units were transfused per patient in the control group and 300 units in patients developing VOD. Platelet requirement persisted a long time after the bone marrow repopulation in VOD patients.

Transfusion ineffectiveness occurred soon after transplantation, often after the first transfusion. The interval between the first and the third transfusions was 5 ± 1 days in VOD patients and 7 ± 2 days in other patients. This difference is statistically significant ($P < 0.05$). No significant difference exists in the recovery at the first transfusion between the two groups ($P < 0.1$). Transfusional recovery remains stable between the first, second, and third transfusions in the control group; however, it sharply drops in patients developing VOD (Fig 1). This difference is statistically significant from the second transfusion onward ($P < 0.02$). Considering together the second and third platelet transfusions after bone marrow transplantation, 9 out of 9 VOD patients have a recovery value less than 25% while only 5 out of 18 do in the control group.

The ineffectiveness of platelet transfusion is a very early symptom of VOD. It was noted at day 6 ± 2 after bone marrow transplantation, while the clinical diagnosis of VOD was made at day 15 ± 5.

Four patients out of 9 (44%) with VOD had anti-HLA antibodies, either prior to or after transplantation. Among the 34 transplanted patients without VOD, 12 (35%) were

Table 2. Criteria of VOD Diagnosis in 13 Patients

	Centrilobular		Congestion of Sinusoid	Liver Biopsy	Days After Therapy*	Echography	Sushepatic Block
	Venocclusion	Fibrosis					
1. JOI	+	+	+	Transjugular	D + 22	+	+
2. MOS	–	+	+	Transparietal	D + 100	+	ND
3. BRI	ND	ND	ND	ND	ND	+	ND
4. DJE	+	+	+	Autopsy	D + 22	+	ND
5. AZI	ND	ND	ND	ND	ND	+	ND
6. BAH	+	+	+	Transjugular	D + 21	+	+
7. VAN	ND	ND	ND	ND	ND	+	ND
8. EZZ	+	+	+	Autopsy	D + 55	ND	ND
9. BOZ	+	+	+	Autopsy	D + 90	ND	ND
10. FAK	–	+	+	Autopsy	D + 62	ND	ND
11. BOU	+	+	+	Autopsy	D + 24	ND	ND
12. DUP	+	+	+	Autopsy	D + 21	ND	ND
13. ABO	+	+	+	Autopsy	D + 17	ND	ND

Clinical features of VOD are initially diagnosed with sudden weight gain and painful hepatomegaly. All patients develop prominant ascitis and jaundice. Biopsies were performed in 10 cases out of 13 for histologic confirmation. Hepatomegaly was confirmed with echography: normality of three sushepatic veins and ascitis were identified in the three remaining patients.

*Days of liver biopsy after therapy.

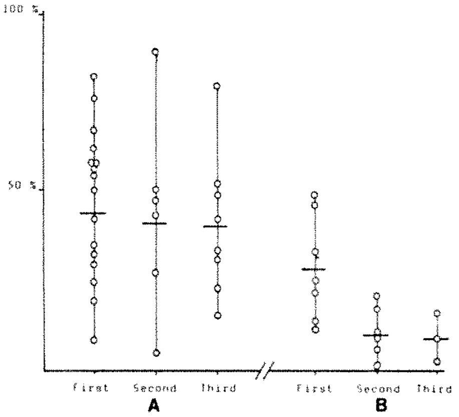

Fig 1. Platelet recovery after transfusion of single-donor platelet concentrates in patients undergoing bone marrow transplantation. (A) Patients without VOD; (B) Patients with VOD.

found to be HLA-immunized in the same conditions. This difference is not significant.

In 4 VOD patients to whom single-donor platelet concentrates from bone marrow donor were transfused, platelet recovery was poor; in three cases the recovery calculated as mentioned previously was 34%, 14%, and 0%, respectively. In the fourth patient, the lifespan was studied by chromium 51-labelled platelets from his bone marrow donor. The platelet half-life was 12 hours; destruction had no preferential site and no splenic or hepatic sequestration was identified.

Coagulation studies performed during the entire period show three different abnormalities. A vitamin K deficiency, almost constant during the first 15 days, is easily corrected with addition of parenteral vitamin K. This deficiency was not more frequent or more intense in either group. In all VOD patients except patient no. 9 a significant rise of FDP is observed. This elevation remains stable during the disease. Factor VIII/von Willebrand activity is elevated in three patients with VOD (680%, 700%, 300%). This elevation was always observed at least 2 times for each patient. Bidimentional chromatography, however, showed no abnormal migratory pattern.

DISCUSSION

Peripheral thrombocytopenia with refractory transfusion was found in all 13 cases of VOD. The exact mechanism involved remains unclear. Peripheral destruction is evidenced by the short life-span (<12 h) of the platelets of the marrow donor as calculated by either transfusion recovery at 16 to 18 h or chromium 51-labelled platelets. The presence of morphologically normal megakaryocytes in the bone marrow smears corroborates this feature. Anti-HLA alloimmunization cannot be held solely responsible since it occurred with the same frequency in both patient groups. Moreover, the ineffectiveness of bone marrow donor platelets, observed in all the 4 cases tested, cannot be explained by an HLA alloimmunization.

We did not look for a specific antiplatelet alloimmunization in this series. These antibodies are responsible for poor platelet transfusion recovery. We think that they are probably not involved in our observation of VOD patients, however, for their occurrence has been shown to be less than 20% in a series of patients undergoing therapeutic aplasia.[22] An autoimmune phenomenon could be suspected; however, it usually appears later after bone marrow transplantation.[23] In our experience, low platelet count invalidates reliability of search for IgG-coated platelets to study autoimmunization.[24] Spleen size was not increased and VOD occurred in a splenectomized patient with the same picture of refractory thrombocytopenia. Splenic or hepatic sequestration was not found in Cr51-labelled platelets.

FDP rise could account for a DIC mechanism in platelet consumption. This rise, however, is never observed before the clinical onset of VOD, while platelet refractoriness is observed earlier. Morever, when the FDP level rises, there is neither concomitant consumption of coagulation factors II, V, and VIII, nor presence of soluble complexes. Thus, the FDP rise cannot be related, at least in the course of the VOD, to a DIC phenomenon and is more likely an early sign of hepatic deficiency.

The concentration of factor VIII/von Willebrand activity was very high. Migration patterns were normal. We have not, however, followed the evolution of the multimers in the surviving patients and multimers could be coated on the platelet in active phase. We cannot, therefore, definitively exclude for the thrombocytopenia a mechanism similar to that described in thrombotic thrombocytopenic purpura or uremic hemolytic syndrome.[25,26]

Few studies in the literature have reported platelet counts during VOD. We have reviewed 9 cases in which this was mentioned: three after 6-thioguanine,[9,10] one during the course of cirrhosis,[7] and one after absorption of Senecio[2] had depressed platelet counts. Normal counts are seen in pulmonary VOD occurring in pregnancy,[27] and in VOD after conditioning with DTIC[19]; however, the pathologic description is ambiguous, because centrolobular veinous thrombosis was present. In the case of Berk et al,[14] the patient died at the 25th day after allogeneic bone marrow transplantation and had a low platelet count, but no information was provided about the frequency of transfusion. Finally, one patient with VOD following autologous bone marrow transplantation reported by Cahn[20] was refractory to transfusion; however, this was ascribed to platelet alloimmunization.

In summary, we retrospectively analyzed 13 cases of VOD occurring after chemotherapy, or autologous or allogeneic bone marrow transplantation. Peripheral thrombocytopenia with refractory transfusion was observed in all cases. The mechanism of refractory thrombocytopenia remains unclear. Nine patients with VOD after allogeneic bone marrow transplantation are compared with the group of allogeneic grafted patients as control. This association is not coincidental, as a significant difference in transfusion requirements exists between patients developing VOD and control patients. Refractoriness to transfusion was noted early in the course of events before establishing the diagnosis of VOD. Low recovery (<25%) at the second and subsequent transfusions should be considered, therefore, as a predictive factor for VOD.

REFERENCES

1. Bras G, Jellife DB, Stuart KL: Veno-occlusive disease of the liver with non-portal type of cirrhosis occurring in Jamaica. Arch Path 57:285, 1954

2. Kumana CR, Lin HJ, Ko W, Wu PC, Tood D: Herbal tea induced hepatic veno-occlusive disease, quantification of toxic alkaloid exposure in adults. Gut 26:101, 1985

3. Stillman AE, Huxtable R, Consroe P, Kohnen P, Smith S: Hepatic veno-occlusive disease due to pyrrolizidine (Senecio) poisoning in Arizona. Gastroenterol 73:349, 1977

4. Tandon BN, Tandon HD, Tandon RK, Narendranathan M, Joshi YK: An epidemia of veno-occlusive disease of liver in central India. Lancet i:271, 1976

5. Safouh M, Shehata A, Elwi A: Hepatic vein occlusion disease in Egyptian children. Arch Path:79:505, 1965

6. Goodman ZD, Ishak KG: Occlusive venous lesions in alcoholic liver disease. A study of 200 cases. Gastroenterol 83:786, 1982

7. Rimarenko S, Jagirdar J: Veno-occlusive syndrome of the liver in an alcoholic man. Mount Sinai J Med 51:292, 1984

8. Gill RA, Onstad GR, Cardamone JM, Maneval DC, Sumner HW: Hepatic veno-occlusive disease caused by 6-Thioguanine. Ann Intern Med 96:58, 1982

9. Griner PF, Elbadawi A, Packman CH: Veno-occlusive disease of the liver after chemotherapy of acute leukemia. Report of two cases. Ann Intern Med 85:578, 1976

10. Krivoy N, Raz R, Carter A, Alroy G: Reversible hepatic veno-occlusive disease and 6-thioguanine. Ann Intern Med 96:788, 1982

11. Marubio AT, Danielson B: Hepatic veino-occlusive disease in a renal transplant patient receiving azathioprine. Gastroenterol 69:739, 1975

12. Shulman HM, McDonald GB, Matthews D, Doney KC, Kopecky KJ, Gauvreau JM, Thomas ED: An analysis of hepatic venocclusive disease and centrilobular hepatic degeneration following bone marrow transplantation. Gastroenterol 79:1178, 1980

13. McDonald GB, Sharma P, Matthews DE, Shulman HM, Thomas ED: Venocclusive disease of the liver after bone marrow transplantation: Diagnosis, incidence and predisposing factors. Hepatology 4:116, 1984

14. Berk PD, Popper H, Krueger GRF, Decter J, Herzig G, Grow RG: Veno-occlusive disease of the liver after allogeneic bone marrow transplantation. Possible association with graft-versus-host disease. Ann Intern Med 90:158, 1979

15. Woods WG, Dehner LP, Nesbit ME, Krivit W, Coccia PF, Ramsay NKC, Kim TH, Kersey JH: Fatal veno-occlusive disease of the liver following high dose chemotherapy, irradiation and bone marrow transplantation. Amer J Med 68:285, 1980

16. Lazarus HM, Gottfried MR, Herzig RH, Phillips GL, Weiner RS, Sarna GP, Fay J, Wolff SN, Sudilowsky O, Gale RP, Herzig GP: Veno-occlusive disease of the liver after high-dose mitomycin C therapy and autologous bone marrow transplantation. Cancer 49:1789, 1982

17. Gottfried MR, Sudilowsky O: Hepatic veno-occlusive disease after high-dose mitomycin C and autologous bone marrow transplantation therapy. Hum Pathol 13:646, 1982

18. McIntyre RE, Magidson JG, Austin GE, Gale RP: Fatal veno-occlusive disease of the liver following high-dose 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) and autologous bone marrow transplantation. Amer J Clin Pathol 75:615, 1981

19. Asbury RF, Rosenthal SN, Descalzi ME, Ratcliffe RL, Arseneau JC: Hepatic veno-occlusive disease due to DTIC. Cancer 45:2670, 1980

20. Cahn JY, Flesh M, Plouvier E, Herve P, Rozenbaum A: Maladie veino-occlusive du foie et autogreffe de moelle osseuse. Role préventif de l'héparine. Nouv Rev Fr Hématol 27:27, 1985

21. Nadler SB, Hidalgo JV, Bloch T: Prediction of blood volume in normal human adults. Surgery 51:224, 1962

22. Fegels JG, Bruynes ECE, Engelfrist CP, Von Den Borne AEG: Serological studies in patients on platelet and granulocyte-substitution therapy. Br J Haematol 52:59, 1982

23. Minchinton RM, Kendra J, Waters AH, Barrett AJ: Autoimmune thrombocytopenia acquired from an allogeneic bone-marrow graft. Lancet ii:627, 1982

24. Eierling P, Vernant JP, Cordonnier C, Rodet M, Maillard JM, Beaujean F, Chevallier AM, Duedari N, Rochant H: Platelet associated IgG after bone marrow transplantation for acute leukemia. Exp Hematol 11:15, 1983 (suppl)

25. Moake JL, Byrnes JJ, Troll JH, Rudy CK, Weinstein MJ, Collanino NM, Hong SL: Abnormal VIII: von Willebrand factor patterns in the plasma of patients with the hemolytic uremic syndrome. Blood 64:592, 1984

26. Moake JL, Rudy CK, Troll JH, Weinstein MJ, Collanino NM, Azocar J, Seder RH, Hong SL, Deykin D: Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombocytic thrombocytopenic purpura. New Engl J Med 307:1432, 1982

27. Tsou E, Waldhorn RE, Kerwin DM, Katz S, Patterson JA: Pulmonary venoocclusive disease in pregnancy. Obstet Gynecol 64:281, 1984

CONCISE REPORT

Chinese $^{A}\gamma$ Fetal Hemoglobin: C to T Substitution at Position −196 of the $^{A}\gamma$ Gene Promoter

By Richard Gelinas, Michael Bender, Carla Lotshaw, Pamela Waber, Haig Kazazian, Jr., and George Stamatoyannopoulos

The molecular basis for the hereditary persistence of fetal hemoglobin (HPFH) phenotype was studied in a Chinese individual who was heterozygous for a nondeletion form of $^{A}\gamma$-HPFH. Both allelic $^{A}\gamma$-globin genes were isolated by molecular cloning and subjected to nucleotide sequence analysis. One $^{A}\gamma$ gene promoter showed a cytosine to thymine transition at position −196, whereas the other promoter was normal. This mutation at position −196 has now been found in unrelated individuals with the $^{A}\gamma$-HPFH phenotype from Italy, Sardinia, and China, suggesting that it may have arisen independently. The implications of this mutation for models of fetal globin gene switching are discussed.

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HEREDITARY persistence of fetal hemoglobin (HPFH) is a condition characterized by continuation of fetal hemoglobin production in the adult in the absence of hematological anomalies.[1,2] Various forms of HPFH have been distinguished on the basis of the levels of Hb F in heterozygotes and the types of globin chains synthesized. Thus, in the common African variant of HPFH, there is no δ- and β-chain synthesis, but production of $^{G}\gamma$ and $^{A}\gamma$ chains of fetal hemoglobin continues in adults.[3-5] In Greek $^{A}\gamma$ HPFH, both $^{A}\gamma$ and β chains are produced from the same chromosome.[6] Two other forms of $^{A}\gamma$ HPFH, the British and Chinese, have been described[7,8]; they have been distinguished from the Greek variant on the basis of levels of Hb F and number of F cells in heterozygotes.[7,8]

Molecular analyses have classified the HPFH mutants into deletion variants, in which δ and β genes are missing while the $^{A}\gamma$ and the $^{G}\gamma$ genes remain intact,[1-5] and the nondeletion mutants, such as the $^{A}\gamma$ HPFH and the $^{G}\gamma$ HPFH variants in which all the genes of the β-globin locus appear to be intact by restriction endonuclease mapping.[4-10] Sequencing of the γ genes of $^{A}\gamma$ HPFH mutants from Greece has revealed a substitution at position −117 of the $^{A}\gamma$ gene promoter,[11,12] and complete concordance has been found between this substitution and the $^{A}\gamma$ HPFH phenotype in Greeks.[13] Sequencing of an $^{A}\gamma$ variant from southern Italy has revealed a substitution at position −196 of the $^{A}\gamma$ gene promoter.[10] In this article we present the results of the sequencing of the Chinese variant of $^{A}\gamma$ HPFH.

MATERIALS AND METHODS

DNA was isolated from peripheral blood lymphocytes as previously described[8] for identification of restriction site polymorphisms in the β-like globin gene clusters[14] and for molecular cloning. The β- through $^{G}\gamma$-globin gene region was isolated from DNA of one of the brothers (II-2) by molecular cloning with a cosmid vector as described in more detail elsewhere.[11] In brief, high mol wt DNA was digested to completion with *Kpn*I and the 39-kilobase (kb) fraction was isolated after velocity sedimentation through gradients of NaCl. This size fraction of DNA was cloned in a cosmid vector, and 120,000 unamplified colonies were screened with ^{32}P-labeled probes to the second intervening sequence of the $^{A}\gamma$ gene. Six positive colonies were isolated and studied by restriction digestion and hybridization with a probe derived from a 0.9-kb *Bam*HI–*Eco*RI fragment derived from the second intervening sequence of the β globin gene.

The 2.6-kb fragment which carries the promoter and 5′ portion of the $^{A}\gamma$ gene was subcloned from cosmids CH1 and CH2 in M13mp18[15] to facilitate sequence analysis by our modification of the primer extension method with dideoxynucleoside triphosphates.[16] The other strand of the $^{A}\gamma$ promoter region was sequenced from the *Stu*I site in the promoter towards the $^{A}\gamma$ gene.

RESULTS

Two adult heterozygotes for the Chinese $^{A}\gamma$ HPFH mutation (individuals II-2 and II-3 of reference 8) were studied. Our aim was to clone the β-globin genomic regions of one of the affected individuals and sequence the γ-globin genes to test whether an abnormality of the $^{A}\gamma$-gene promoter was present. To distinguish between the two allelic β-globin genomic regions, DNA from the two affected individuals was digested with several restriction endonucleases in search of a heterozygosity in one restriction endonuclease site. Individuals II-2 and II-3 proved to be heterozygous for the *Ava*II site located in the second intron of the β-globin gene.[17] This polymorphism facilitated the subsequent identification of cosmids containing each of the two β-globin gene clusters.

The previously described cosmid cloning strategy and vector[11] were used to isolate the 39-kb portion of the β-like globin gene cluster, which includes the entire $^{G}\gamma$ to β-globin gene region.[18] An *Ava*II digestion of the DNA from three cosmid clones isolated independently revealed that two cosmids (cosmids CH1 and CH3) contained the *Ava*II site in the β gene, whereas one cosmid (CH2) lacked this site (Fig 1).

DNA from cosmids CH1 and CH2 was digested with *Eco*RI, and the 2.6-kb fragment which contains the $^{A}\gamma$ gene promoter was subcloned in M13 vectors for sequence analy-

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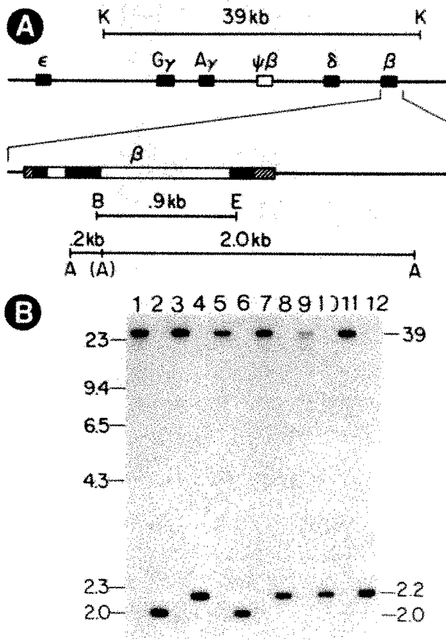

Fig 1. Cloning strategy and detection of AvaII polymorphism. Top: the 39-kilobase (kb) *KpnI* fragment which carries the $^{G}\gamma$ through β-gene region was isolated by cloning in a cosmid vector as described.[11] The *BamHI* to *EcoRI* restriction fragment 0.9 kb in length used to reveal the *AvaII* polymorphism is shown along with the AvaII map in the region of the β gene. Bottom: DNA from six independently isolated cosmid clones was digested with *KpnI* (lanes 1, 3, 5, 7, 9, and 11) or *AvaII* (lanes 2, 4, 6, 8, 10 and 12), fractionated on an agarose gel, and transferred to a nitrocellulose filter. The filter was probed with the 0.9-kb fragment, and the presence of the polymorphic *AvaII* site results in detection of a 2.0-kb band (cosmids CH1 and CH3, lanes 2 and 6), whereas in the absence of the site, a 2.2-kb band is found (cosmid CH2, lane 4). K, B, E, and A stand for recognition sites for the restriction enzymes *KpnI*, *BamHI*, *EcoRI*, and *AvaII*, respectively.

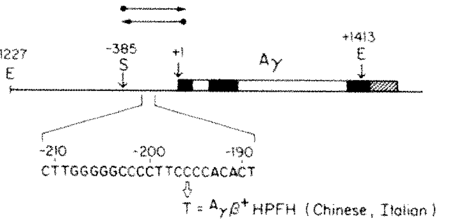

Fig 2. Structure of the $^{A}\gamma$ promoter in Chinese $^{A}\gamma$ HPFH. Numbers refer to bases relative to the start of transcription as +1. E and S: *EcoRI* and *SphI* cleavage sites. Horizontal arrows indicate direction and extent of nucleotide sequencing reactions.

sis. The nucleotide sequence was determined for each of the two allelic $^{A}\gamma$ genes over the region −400 through +25 relative to start of the mRNA. The sequence of the promoter from CH1 was that of a normal $^{A}\gamma$ globin gene. The sequence from CH2 had a C to T substitution at position −196 relative to the residue at which transcription starts (Fig 2). This result was confirmed by sequencing the other strand of the 2.6-kb *EcoRI* fragment from CH2 through this region.

Both $^{A}\gamma$ genes proved to be of the "A-type" as described by Slightom and co-workers[19] and Shen and colleagues.[20] No other differences were found between the sequences of CH1 or CH2 and the reported sequence of a normal A-type $^{A}\gamma$ gene.

DISCUSSION

In this study, we sequenced the promoter regions of the two allelic $^{A}\gamma$ genes of a Chinese individual with nondeletion $^{A}\gamma$ HPFH. We found that the sequence of one $^{A}\gamma$-gene promoter was normal, whereas the promoter of the allelic $^{A}\gamma$ gene contained a C to T substitution at position −196. This substitution has been previously found in the promoter of the HPFH chromosome of an Italian family with $^{A}\gamma$ HPFH.[21] We have no formal genetic evidence that the −196 C to T substitution is indeed located on the Chinese $^{A}\gamma$ HPFH chromosome, but the association of this mutation with the Italian $^{A}\gamma$ HPFH makes it likely that the substitution at position −196 is the underlying lesion in the Chinese $^{A}\gamma$ HPFH as well. Haplotype analyses have shown that the Italian $^{A}\gamma$ HPFH is associated with haplotype V.[14] We found that the Chinese $^{A}\gamma$ HPFH was associated with haplotype I. These results and the ethnic origin of the probands suggest that the Italian and Chinese $^{A}\gamma$ HPFH arose as independent mutations. Other mutations of the β-globin cluster including the β^{S}, β^{E}, and certain β-thalassemia alleles also have probably had independent origins.[22]

The point mutation at −196 occurs in yet a third genetic background. The Sardinian form of $\delta\beta$-thalassemia is associated with absence of β-chain synthesis and continued $^{A}\gamma$-chain synthesis in *cis*.[10] It is now apparent that the *cis* β-chain deficiency is due to the presence of a nonsense mutation at codon 39,[23,24] whereas the continued $^{A}\gamma$ expression is due to a C to T substitution at −196 of the promoter of the $^{A}\gamma$ gene of the $\delta\beta$ thalassemic chromosome.[25]

The existence of base substitution mutations at position −196 in $^{A}\gamma$ HPFH and −202 in $^{G}\gamma$ HPFH[25] suggests that the −200 region of the γ-globin genes is involved in the control of γ-gene expression during development. In support of this is the finding of a substitution at position −198 of the $^{A}\gamma$ gene of the British $^{A}\gamma$ HPFH (D. Weatherall, personal communication). Recent evidence also shows that a mutation at position −196 or −202 is sufficient to either alter or abolish the S1 nuclease hypersensitive site which is normally present at position −208 to −216 in supercoiled plasmids in vitro (W.J. Gray and T.J. Ley, personal communication). The −200 region may be the site of interaction between the γ-globin gene and *trans* acting elements which turn off the γ genes in the perinatal period. This interaction may be weakened by the substitutions −196, −198, and −202, resulting in continuation of Hb F production and the phenotype.

Assays can now be developed to test the association of these point mutations with the $^{A}\gamma$ or $^{G}\gamma$ ndHPFH phenotype. Quantitation of $^{A}\gamma$ transcription from a normal promoter or from a promoter carrying a point mutation at −117, −196, or −202 in transgenic mice or cultured erythroid cells may help answer these questions.

REFERENCES

1. Weatherall D, Clegg J: The Thalassemia Syndromes. Blackwell, Oxford, third ed 1981

2. Collins F, Weissman, S: Prog Nucleic Acids Res Mol Biol 31:315, 1984

3. Fritsch E, Lawn R, Maniatis T: Nature 279:598, 1979

4. Tuan D, Murnane M, DeRiel J, Forget B: Nature 258:335, 1980

5. Bernards R, Flavell R: Nucleic Acids Res 8:1521, 1980

6. Sofroniadou K, Wood WG, Nute PE, Stamatoyannopoulos G: Br J Haematol 29:137, 1975

7. Jones R, Old J, Wood W, Clegg J, Weatherall D: Br J Haematol 50:415, 1982

8. Farquhar M, Gelinas R, Tatsis B, Murray J, Yagi M, Mueller R, Stamatoyannopoulos G: Am J Hum Genet 35:611, 1983

9. Basley J, Rappaport E, Schwartz E, Surrey S: Blood 59:828, 1982

10. Ottolenghi S, Giglioni B, Taramelli R, Comi P, Mazza U, Saglio G, Camaschella C, Izzo P, Cao A, Galanello R, Gimferrer E, Baiget M, Gianni A: Proc Natl Acad Sci USA 79:2347, 1982

11. Gelinas R, Endlich B, Pfeiffer C, Yagi M, Stamatoyannopoulos G: Nature 313:323, 1985

12. Collins F, Metherall J, Yamakawa M, Pan J, Weissman S, Forget B: Nature 313:325, 1985

13. Waber P, Bender M, Gelinas R, Kattamis C, Karaklis A, Sofroniadou K, Stamatoyannopoulos G, Collins F, Forget B, Kazazian H: Blood (in press)

14. Orkin S, Kazazian H, Antonarakis S, Goff S, Boehm C, Sexton J, Waber P, Giardina P: Nature 296:627, 1982

15. Norrander J, Kempe T, Messing J: Gene 26:101, 1983

16. Sanger F, Nicklen S, Coulson A: Proc Natl Acad Sci USA 74:5463, 1977

17. Antonarakis S, Boehm C, Giardina P, Kazazian H: Proc Natl Acad Sci USA 79:137, 1982

18. Van der Ploeg L, Konings A, Oort M, Roos L, Bernini L, Flavell R: Nature 283:637, 1980

19. Slightom J, Blechl A, Smithies O: Cell 21:627, 1980

20. Shen S, Slightom J, Smithies O: Cell 26:191, 1980

21. Giglioni B, Casini C, Mantovani R, Merli S, Comi R, Ottolenghi S, Sagio G, Camaschela C, Mazza U: EMBO J 3:2641, 1984

22. Orkin S, Kazazian H: Annu Rev Genet 18:131, 1984

23. Cao A, Melia M, Galanello R, Angius A, Furbetta M, Giordano P, Bernini L: J Med Genet 19:184, 1982

24. Pirastu M, Kan Y, Galanello R, Cao A: Science 223:929, 1984

25. Ottolenghi S, Giglioni B, Comi P, Pulazzini A, Merli S, Casini C, Mantovani R, Camaschella C, Saglio G, in Koprowski H, Ferrone S, Albertini A (eds): Biotechnology in Diagnostics—Symposia of the Giovanni Lorenzini Foundation, Amsterdam, Elsevier, 1985, p 269

CORRESPONDENCE

CHEMOTAXIS OF CORD BLOOD GRANULOCYTES

To the Editor:

Nunoi et al recently suggested that the impaired chemotaxis of cord blood granulocytes (CBG) might be explained, at least in part, by a decreased number of receptors for chemotactic factors plus abnormalities of receptor recycling.[1] This suggestion should be considered with great caution for three reasons. First, not all investigators agree that receptors for chemotactic factors are decreased in number on neonatal granulocytes. The authors cite an earlier paper[2] in which they demonstrated decreased numbers of chemotactic receptors when data from a single neonate were subjected to Scatchard analysis. They studied five additional neonates using a simple one-point assay and found decreased chemotactic peptide binding. Thus, they evaluated only a few infants and failed to cite work performed by two other groups who found normal binding of chemotactic factors by neonatal granulocytes.[3,4]

Second, the decrease in the digestive activity of lysates from CBG, which was used by Nunoi et al as an indicator of the receptor recycling process, was not very convincing.[1] Although the mean value was statistically decreased, only one of nine CBG samples had a value below the complete range of values for granulocytes obtained from adult subjects (reference 1, Fig 10). Third, the biologic significance of decreased digestive activity, if any, as it pertains to chemotaxis has not been defined.

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REFERENCES

1. Nunoi H, Endo F, Chikazawa S, Matsuda I: Regulation of receptors and digestive activity toward synthesized formyl-chemotactic peptide in human polymorphonuclear leukocytes. Blood 66:106, 1985

2. Nunoi H, Endo F, Chikazawa S, Namikawa T, Matsuda I: Chemotactic receptor of cord blood granulocytes to the synthesized chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine. Pediatr Res 17:57, 1983

3. Anderson DC, Hughes BJ, Smith CW: Abnormal mobility of neonatal polymorphonuclear leukocytes: Relationship to impaired redistribution of surface adhesion sites by chemotactic factor or colchicine. J Clin Invest 68:863, 1981

4. Strauss RG, Snyder EL: Chemotactic peptide binding by intact neutrophils from human neonates. Pediatr Res 18:63, 1984

HTLV-III ANTIBODY STATUS IN HOUSEHOLD CONTACTS OF SEROPOSITIVE HEMOPHILIACS

To the Editor:

We read with interest the report by Lawrence et al[1] about the HTLV-III antibody status of household contacts of hemophilic patients. We have obtained similar data in relatives of seropositive hemophilic children living in the same households and actively participating in the care of these children. Most of the children were on home treatment and concentrates were regularly administered by their parents or by the patients themselves with the help of their parents. HTLV-III antibodies were determined by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blotting. For comparison, we determined the serologic marker for hepatitis B infection. With the exception of actively immunized persons, a person was considered to have evidence for hepatitis B infection when one of the markers (HBsAg, HBeAg, antiHBs, antiHBc, or antiHBe) was positive.

Forty-five persons living in 17 households with 18 HTLV-III antibody-positive hemophilic children were investigated. Sixteen of the hemophilic children were also positive for hepatitis B. HTLV-III antibodies were detected in none of the relatives. In contrast, four of 43 relatives of hepatitis B positive children were hepatitis B positive, ie, 9.3% of the relatives of hemophilic children were hepatitis B positive. In a study comprising all our hepatitis B positive hemophilic children, the prevalence in relatives was even higher and certainly higher than in our general population.[2,3] Our data support the general impression that the risk of contracting hepatitis B is greater than that of contracting HTLV-III from seropositive patients.

There is great concern in our general population about the risk of infection by HTLV-III antibody-positive patients despite the accumulating evidence that the chance of cross-infection by casual contact or even by providing medical services to these patients is very small.[4] Social integration is difficult for hemophilic children attending day care centers, kindergarten, or school. The data obtained from persons living in close contact with hemophilic children should help to facilitate their social integration.

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REFERENCES

1. Lawrence DN, Jason JM, Bouhasin JD, McDougal JS, Knutsen AP, Evatt BL, Joist JH: HTLV-III/LAV antibody status of spouses and household contacts assisting in home infusion of hemophilia patients. Blood 66:703, 1985

2. Deutsch J, Fueger GF, Muntean W: The incidence of hepatitis B virus infections in families of haemophilic children. J Hepatol 2:221, 1985 (suppl)

3. Lorant P, Knapp E, Hoffman H, Frisch-Niggemeyer W, Pichler H: Die Hepatitis B-Durchseuchung des Wiener Krankenanstaltenpersonals. Wien Klin Wochenschr 95:235, 1983

4. Jones P, Hamilton P: HTLV-III antibodies in haematology staff. Lancet i:217, 1985

To the Editor:

Dr Muntean and colleagues have provided further evidence for the extreme infrequency of seropositivity to HTLV-III/LAV in family members of seropositive hemophiliacs. Muntean et al have also demonstrated that, to date, the families of hemophiliacs are at greater risk for hepatitis B infection than HTLV-III/LAV infection.

Hepatitis B infection is a recognized and essentially inevitable consequence of hemophilia therapy, based on observation of users of currently available products. In spite of serologic screening of donated plasma to detect evidence of past infection with hepatitis B, factor concentrate therapy still results in hepatitis B infection for most hemophiliacs. Depending on the duration of their hepatitis B antigenemia, long-term recipients of factor concentrates may be a source of intrahousehold spread to hepatitis B for relatively long periods.[1]

Only since the relatively recent introduction of HTLV-III/LAV into the hemophilic population, predominantly via factor concentrates, has the risk of household spread of this infection become a serious possibility for this population. Long-term studies in populations as described by Dr Muntean offer a means of quantifying the risk per year of acquiring HTLV-III/LAV infection due to contact with hemophiliacs. The potentially ominous consequences of infection with HTLV-III/LAV make the extreme rarity of casual interpersonal transmission, and the rarity of accidental needle-stick transmission by those assisting in infusion,[2] very welcome news.

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REFERENCES

1. Bernier RH, Sampliner R, Gerety R, Tabor E, Hamilton F, Nathanson N: Hepatitis B infection in households of chronic carriers of hepatitis B surface antigen: Factors associated with prevalence of infection. Am J Epidemiol 116:199, 1982

2. Centers for Disease Control: Recommendations for preventing transmission of infection with HTLV-III/LAV in the workplace. MMWR 34:681, 1985

ERRATUM

In the article entitled "Clonal Rearrangement and Expression of the T Cell Receptor β Gene and Involvement of the Breakpoint Cluster Region in Blast Crisis of CGL" by Chan et al, which appeared in the February 1986 issue (Volume 67, Number 2, page 533), Figure 2 was not clearly reproduced. It is reprinted below.

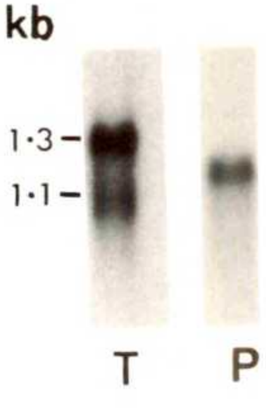

Fig 2. Autoradiography of RNA transcripts hybridized with a TCR β constant region probe. T, thymocyte RNA: P, patient RNA. Sizes of RNA are given in kilobases (KB).

ANNOUNCEMENTS

INTERNATIONAL CONGRESS ON THE BIOLOGICAL AND CLINICAL ASPECTS OF PHAGOCYTE FUNCTION

Sponsored by the Italian Society of Hematology
Chairmen: Carlo Mauri and Salvatore Carlo Rizzo

September 7–10, 1986
Pavia, Italy

Università di Pavia   Policlinico S. Matteo—Pavia—IRCCS

Scientific program topics: Leukocyte random locomotion, chemotaxis, and chemokinesis; membrane metabolism; pharmacological manipulation and immunomodulation of phagocyte function; the role of phagocytes in inflammation; leukotrienes, prostaglandins, chemotaxis, and phagocyte function; membrane chemotactic receptor interactions; enkephalins, endorphins, and phagocyte function; immunodeficiency and phagocyte function; and clinical aspects of phagocyte disorders.

The official languages of the Congress will be English and Italian. Simultaneous translation will be provided. Registration fees, including taxes, are: US $200; for Italian participants, Lit. 300.000; for SIE members, Lit. 250.000. Fees should be paid by check to the Organizing Secretariat: Centro Italiano Congressi CIC, p.zza A. Diaz 6, 20123 Milano, Italy, and will give right to access to the Conference Room, a copy of the Abstract book, working lunches during the Congress, and a copy of the Proceedings. The Abstracts will be published in a special issue of *Hematology Reviews and Communications*. The Conference Proceedings (main lectures and some selected communications in extenso) will be published by an international publisher.

THE THIRD WILLIAM B. CASTLE SYMPOSIUM ON ADVANCES IN HEMATOLOGY

Friday, October 24, 1986
Grand Ballroom, Copley Plaza Hotel
Boston, Massachusetts

Guest speakers:		
	Richard Cooper (Milwaukee)	Ralph Nachman (New York)
	Robert Gallo (Bethesda)	Saul Rosenberg (Stanford)
	Philip Leder (Boston)	Sol Sherry (Philadelphia)

The Castle Symposium is sponsored by the West Roxbury Veterans Administration Hospital, Brigham and Women's Hospital, and Harvard Medical School. Advance registration is required. For further information and registration forms, please contact Andrew I. Schafer, MD, Hematology Division, Brigham and Women's Hospital, 75 Francis St, Boston, MA 02115. Telephone (617)732-5840.

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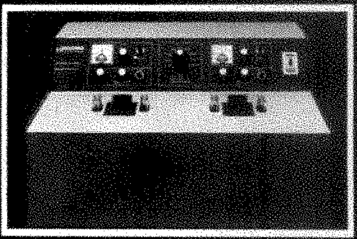
*Thrombosis & Haemostasis Vol. 51-2 (154-156) 1984.



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A Department of Hematologic Oncology has recently been established at Roswell Park Memorial Institute. This Department has a major interest in myeloid leukemia and related disorders. In addition, there will be a major program exploring the treatment of advanced solid tumors through the use of new and novel therapies in conjunction with autologous bone marrow transplantation. An extensive laboratory program involving the development and testing of drug sensitivity assays, the assessment of the cell cycle characteristics of neoplastic cells in vitro, and of proto-oncogene expression in vivo and in vitro are closely integrated with the clinical program.

A position is available in this department for a physician who has completed his or her training in Medical Oncology and/or Hematology who has an interest in both clinical trials and laboratory science. This is a tenure tract position. An Equal Opportunity/Affirmative Action Employer. Send curriculum vitae/resumes to Dr. Harvey Preisler, Department of Hematologic Oncology, 666 Elm Street, Buffalo, NY 14263.

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